A10

145 (new). A composition comprising the antibody of claim 143, and a pharmaceutically acceptable carrier.

REMARKS

The title has been amended to recite "ANTIBODIES TO VERTEBRATE DELTA PROTEINS AND FRAGMENTS" such that the title is clearly indicative of the claimed subject matter.

The specification has been amended to reflect that the present application is a divisional application of Application Serial No. 08/981,392, filed December 22, 1997. The specification has also been amended to incorporate the correct sequence identifiers pursuant to 37 C.F.R. § 1.821(d) and the Sequence Listing submitted herewith pursuant to 37 C.F.R. § 1.821(c).

The specification has been amended at page 29, lines 26, 28 and 33, and at page 30, line 21 to insert the same material as that incorporated by reference, in accordance with M.P.E.P 2163.07(b) and 608.01(p). No new matter has been introduced. Specifically, Kozbor et al., 1983, Immunology Today 4:72 (a copy of which is attached hereto as Exhibit A) was incorporated by reference at page 29, lines 26 and 33 of the specification with respect to the description, at page 29, lines 23-30, for types of production of antibody molecules by continuous cell lines. The actual text of the first sentence of the Abstract at page 72, and the actual text of the first two sentences of the section entitled *Selection of antigen-specific cells* on page 76, left column have been inserted into the specification. Additionally, Kohler and Milstein, 1975, Nature 256:495 (a copy of which is attached hereto as Exhibit B) was incorporated by reference at page 29, line 28 of the specification with respect to the description, at page 29, lines 23-30, for types of production of antibody molecules by continuous cell lines. The actual text of a sentence on page 495, left column, has been inserted into the specification. Further, Huse et al., 1989, Science 246:1275-1281 (a copy of which is attached hereto as Exhibit C) was incorporated by reference at page 30, line 21 of the specification with respect to the description, at page 30, lines 13-21, for the production of phage Fab expression libraries. The same subject matter as in the text from page 1277, left column, to page 1278, right column, has been inserted into the specification.

Applicants submit herewith a Declaration under 37 C.F.R. § 1.68 and M.P.E.P. 608.01(p) which states that the amendatory material that was included in the specification at page 29, lines 26, 28 and 33 consists of the same material incorporated by

reference in the application as filed at page 29, lines 23-30, and that the amendatory material that was included in the specification at page 30, line 21 consists of the same material incorporated by reference in the application as filed at page 30, lines 13-21.

Upon entry of this amendment, claims 29-32, 60, 61 and 99-145 will be pending. Claims 29-32, 60 and 61 have been amended, and new claims 99-145 have been added, to more particularly point out and distinctly claim the subject matter of the present invention. Specifically, claims 29, 60 and 61 have been amended to recite that the antibody binds a vertebrate Delta protein, which Delta protein is encoded by a first nucleic acid that hybridizes under low stringency conditions to a second nucleic acid, or its complement, which second nucleic acid is selected from the group consisting of the chick *Delta* sequence of SEQ ID NO:1, the antisense strand to the chick *Delta* sequence of SEQ ID NO:1, the chick *Delta* sequence of SEQ ID NO:3, the antisense strand to the chick *Delta* sequence of SEQ ID NO:3, the mouse *Delta* sequence of SEQ ID NO:11, the antisense strand to the mouse *Delta* sequence of SEQ ID NO:11, the human *Delta* sequence of SEQ ID NO:14, the antisense strand to the human *Delta* sequence of SEQ ID NO:14, the human *Delta* sequence of SEQ ID NO:26, the antisense strand to the human *Delta* sequence of SEQ ID NO:26, the mouse-human *Delta* consensus sequence of SEQ ID NO:24, and the antisense strand to the mouse-human *Delta* consensus sequence of SEQ ID NO:24, said low stringency conditions comprising pretreatment for 6 hours at 40°C in a solution containing 35% formamide, 5X SSC, 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 0.1% PVP, 0.1% Ficoll, 1% BSA, and 500 µg/ml denatured salmon sperm DNA; hybridization for 18-20 hours at 40°C in a solution containing 35% formamide, 5X SSC, 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 0.02% PVP, 0.02% Ficoll, 0.2% BSA, 100 µg/ml denatured salmon sperm DNA, and 10% (wt./vol.) dextran sulfate; washing for 1.5 hours at 55°C in a solution containing 2X SSC, 25 mM Tris-HCl (pH 7.4), 5 mM EDTA and 0.1% SDS; and a second washing for 1.5 hours at 60°C in a solution containing 2X SSC, 25 mM Tris-HCl (pH 7.4), 5 mM EDTA and 0.1% SDS, and which antibody does not bind a *Drosophila* Delta protein. Support for this amendment is found in the specification as filed, *inter alia*, at page 12, lines 14-15; page 13, line 33 to page 15, line 3; page 28, line 31 to page 29, line 4; page 30, line 22 to page 31, line 3; and Figures 1, 7, 10 and 12.

Claim 30 has been made independent and recites an antibody, which binds a human Delta protein, which Delta protein is encoded by a first nucleic acid that hybridizes under low stringency conditions to a second nucleic acid, or its complement, which second

nucleic acid is selected from the group consisting of the human *Delta* sequence of SEQ ID NO:14, the antisense strand to the human *Delta* sequence of SEQ ID NO:14, the human *Delta* sequence of SEQ ID NO:26, and the antisense strand to the human *Delta* sequence of SEQ ID NO:26, said low stringency conditions comprising pretreatment for 6 hours at 40°C in a solution containing 35% formamide, 5X SSC, 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 0.1% PVP, 0.1% Ficoll, 1% BSA, and 500 µg/ml denatured salmon sperm DNA; hybridization for 18-20 hours at 40°C in a solution containing 35% formamide, 5X SSC, 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 0.02% PVP, 0.02% Ficoll, 0.2% BSA, 100 µg/ml denatured salmon sperm DNA, and 10% (wt./vol.) dextran sulfate; washing for 1.5 hours at 55°C in a solution containing 2X SSC, 25 mM Tris-HCl (pH 7.4), 5 mM EDTA and 0.1% SDS; and a second washing for 1.5 hours at 60°C in a solution containing 2X SSC, 25 mM Tris-HCl (pH 7.4), 5 mM EDTA and 0.1% SDS, which does not bind a *Drosophila* Delta protein. Support for this amendment is found in the specification as filed, *inter alia*, at page 12, lines 14-15; page 13, line 33 to page 15, line 3; page 28, line 31 to page 29, line 4; page 30, line 22 to page 31, line 3; and Figures 10 and 12. Claim 31 has been amended to depend from claim 29 or 30. Claim 32 has been amended to recite that the fragment of the antibody binds a vertebrate Delta protein. Support for this claim is found in the specification at page 28, lines 31-34.

New claims 99-145 have been added. Support for the newly added claims is set forth in the table below.

CLAIM	SUPPORT IN THE SPECIFICATION
99, 126	page 25, line 8 to page 26, line 24; Figures 2, 8, 11 and 14
100, 123	page 29, lines 23-26
101	page 30, lines 22-30
102-104, 124, 143	page 31, lines 13-15; page 60, lines 19-20; page 64, lines 15-18
109, 114, 129	page 12, lines 14-15, page 13, line 33 to page 15, line 3; page 28, line 31 to page 31, line 15; Figures 1, 7, 10 and 12
105, 110, 128, 139	page 12, lines 14-15, page 13, line 33 to page 15, line 3; page 28, line 31 to page 31, line 15; Figure 14; Section 8 (pages 76-70)

106, 111, 127, 140	page 12, lines 14-15, page 13, line 33 to page 15, line 3; page 28, line 31 to page 31, line 15; Figure 10; Section 8 (pages 76-79)
107, 108, 113, 138	page 13, line 33 to page 15, line 3, Figures 10, 12
112, 141	page 12, lines 14-15, page 13, line 33 to page 15, line 3; page 28, line 31 to page 31, line 15; Figure 7; Section 7 (pages 74-75)
115, 116, 118-120	page 25, lines 24-32; page 30, lines 33-37; page 36, line 24 to page 37, line 23
117	page 33, lines 11-33
121, 122, 142	page 28, line 31 to page 31, line 15
125, 144, 145	page 31, lines 13-15; page 63, line 18 to page 64, line 18
130	page 35, lines 6-12
131-137	page 12, lines 14-15, page 13, line 33 to page 15, line 3; page 28, line 31 to page 31, line 15; Figures 1, 7, 10 and 12; and in the text that was inserted at page 26, lines 26, 29 and 33 and at page 30, line 21 by amendment herein

None of the above-made amendments constitute new matter under 35 U.S.C. § 132.

CONCLUSION

Applicants respectfully request that the above-made amendments and remarks of the present preliminary amendment be entered and made of record in the file history of this application.

Respectfully submitted,

Date: February 15, 2001

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CLAIMS THAT WILL BE PENDING UPON ENTRY OF PRELIMINARY AMENDMENT

29. An antibody which binds a vertebrate Delta protein, which Delta protein is encoded by a first nucleic acid that hybridizes under low stringency conditions to a second nucleic acid, or its complement, which second nucleic acid is selected from the group consisting of the chick *Delta* sequence of SEQ ID NO:1, the antisense strand to the chick *Delta* sequence of SEQ ID NO:1, the chick *Delta* sequence of SEQ ID NO:3, the antisense strand to the chick *Delta* sequence of SEQ ID NO:3, the mouse *Delta* sequence of SEQ ID NO:11, the antisense strand to the mouse *Delta* sequence of SEQ ID NO:11, the human *Delta* sequence of SEQ ID NO:14, the antisense strand to the human *Delta* sequence of SEQ ID NO:14, the human *Delta* sequence of SEQ ID NO:26, the antisense strand to the human *Delta* sequence of SEQ ID NO:26, the mouse-human *Delta* consensus sequence of SEQ ID NO:24, and the antisense strand to the mouse-human *Delta* consensus sequence of SEQ ID NO:24, said low stringency conditions comprising pretreatment for 6 hours at 40°C in a solution containing 35% formamide, 5X SSC, 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 0.1% PVP, 0.1% Ficoll, 1% BSA, and 500 µg/ml denatured salmon sperm DNA; hybridization for 18-20 hours at 40°C in a solution containing 35% formamide, 5X SSC, 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 0.02% PVP, 0.02% Ficoll, 0.2% BSA, 100 µg/ml denatured salmon sperm DNA, and 10% (wt./vol.) dextran sulfate; washing for 1.5 hours at 55°C in a solution containing 2X SSC, 25 mM Tris-HCl (pH 7.4), 5 mM EDTA and 0.1% SDS; and a second washing for 1.5 hours at 60°C in a solution containing 2X SSC, 25 mM Tris-HCl (pH 7.4), 5 mM EDTA and 0.1% SDS, and which antibody does not bind a *Drosophila* Delta protein.

30. An antibody, which binds a human Delta protein, which Delta protein is encoded by a first nucleic acid that hybridizes under low stringency conditions to a second nucleic acid, or its complement, which second nucleic acid is selected from the group consisting of the human *Delta* sequence of SEQ ID NO:14, the antisense strand to the human *Delta* sequence of SEQ ID NO:14, the human *Delta* sequence of SEQ ID NO:26, and the antisense strand to the human *Delta* sequence of SEQ ID NO:26, said low stringency conditions comprising pretreatment for 6 hours at 40°C in a solution containing 35% formamide, 5X SSC,

50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 0.1% PVP, 0.1% Ficoll, 1% BSA, and 500 µg/ml denatured salmon sperm DNA; hybridization for 18-20 hours at 40°C in a solution containing 35% formamide, 5X SSC, 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 0.02% PVP, 0.02% Ficoll, 0.2% BSA, 100 µg/ml denatured salmon sperm DNA, and 10% (wt./vol.) dextran sulfate; washing for 1.5 hours at 55°C in a solution containing 2X SSC, 25 mM Tris-HCl (pH 7.4), 5 mM EDTA and 0.1% SDS; and a second washing for 1.5 hours at 60°C in a solution containing 2X SSC, 25 mM Tris-HCl (pH 7.4), 5 mM EDTA and 0.1% SDS, which does not bind a *Drosophila* Delta protein.

31. The antibody of claim 29 or 30 which is monoclonal.

32. A molecule comprising a fragment of the antibody of claim 31, which fragment binds a vertebrate Delta protein.

60. A composition comprising an amount of an antibody which binds to a vertebrate Delta protein; and a pharmaceutically acceptable carrier, which Delta protein is encoded by a first nucleic acid that hybridizes under low stringency conditions to a second nucleic acid, or its complement, which second nucleic acid is selected from the group consisting of the chick *Delta* sequence of SEQ ID NO:1, the antisense strand to the chick *Delta* sequence of SEQ ID NO:1, the chick *Delta* sequence of SEQ ID NO:3, the antisense strand to the chick *Delta* sequence of SEQ ID NO:3, the mouse *Delta* sequence of SEQ ID NO:11, the antisense strand to the mouse *Delta* sequence of SEQ ID NO:11, the human *Delta* sequence of SEQ ID NO:14, the antisense strand to the human *Delta* sequence of SEQ ID NO:14, the human *Delta* sequence of SEQ ID NO:26, the antisense strand to the human *Delta* sequence of SEQ ID NO:26, the mouse-human *Delta* consensus sequence of SEQ ID NO:24, and the antisense strand to the mouse-human *Delta* consensus sequence of SEQ ID NO:24, said low stringency conditions comprising pretreatment for 6 hours at 40°C in a solution containing 35% formamide, 5X SSC, 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 0.1% PVP, 0.1% Ficoll, 1% BSA, and 500 µg/ml denatured salmon sperm DNA; hybridization for 18-20 hours at 40°C in a solution containing 35% formamide, 5X SSC, 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 0.02% PVP, 0.02% Ficoll, 0.2% BSA, 100 µg/ml denatured salmon sperm DNA, and 10% (wt./vol.) dextran sulfate; washing for 1.5 hours at 55°C in a solution containing 2X SSC, 25 mM Tris-HCl (pH 7.4), 5 mM EDTA and 0.1% SDS; and a

second washing for 1.5 hours at 60°C in a solution containing 2X SSC, 25 mM Tris-HCl (pH 7.4), 5 mM EDTA and 0.1% SDS, and which antibody does not bind a *Drosophila* Delta protein.

61. A composition comprising an amount of a fragment or derivative of an antibody to a vertebrate Delta protein containing the binding domain of the antibody; and a pharmaceutically acceptable carrier, which Delta protein is encoded by a first nucleic acid that hybridizes under low stringency conditions to a second nucleic acid, or its complement, which second nucleic acid is selected from the group consisting of the chick *Delta* sequence of SEQ ID NO:1, the antisense strand to the chick *Delta* sequence of SEQ ID NO:1, the chick *Delta* sequence of SEQ ID NO:3, the antisense strand to the chick *Delta* sequence of SEQ ID NO:3, the mouse *Delta* sequence of SEQ ID NO:11, the antisense strand to the mouse *Delta* sequence of SEQ ID NO:11, the human *Delta* sequence of SEQ ID NO:14, the antisense strand to the human *Delta* sequence of SEQ ID NO:14, the human *Delta* sequence of SEQ ID NO:26, the antisense strand to the human *Delta* sequence of SEQ ID NO:26, the mouse-human *Delta* consensus sequence of SEQ ID NO:24, and the antisense strand to the mouse-human *Delta* consensus sequence of SEQ ID NO:24, said low stringency conditions comprising pretreatment for 6 hours at 40°C in a solution containing 35% formamide, 5X SSC, 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 0.1% PVP, 0.1% Ficoll, 1% BSA, and 500 µg/ml denatured salmon sperm DNA; hybridization for 18-20 hours at 40°C in a solution containing 35% formamide, 5X SSC, 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 0.02% PVP, 0.02% Ficoll, 0.2% BSA, 100 µg/ml denatured salmon sperm DNA, and 10% (wt./vol.) dextran sulfate; washing for 1.5 hours at 55°C in a solution containing 2X SSC, 25 mM Tris-HCl (pH 7.4), 5 mM EDTA and 0.1% SDS; and a second washing for 1.5 hours at 60°C in a solution containing 2X SSC, 25 mM Tris-HCl (pH 7.4), 5 mM EDTA and 0.1% SDS, and which antibody does not bind a *Drosophila* Delta protein.

Please add the following new claims:

99. The antibody of claim 29, 60 or 61, in which the Delta protein comprises an amino acid sequence selected from the group consisting of the chick Delta sequence of SEQ ID NO:2, the mouse Delta sequence of SEQ ID NO:12, the human Delta sequence of SEQ ID NO:23, and the human Delta sequence of SEQ ID NOS:65-80.

100. The composition of claim 60 or 61, in which the antibody is monoclonal.

101. A fragment of the antibody of claim 29 or 30, which fragment binds a vertebrate Delta protein.

102. The antibody of claim 29, 30, 31 or 99, which antibody is purified.

103. The fragment of claim 101, which fragment is purified.

104. The molecule of claim 32, which molecule is purified.

105. The antibody of claim 29, in which the Delta protein comprises the amino acid sequence of SEQ ID NOS:65-80.

106. The antibody of claim 29, in which the Delta protein comprises the amino acid sequence of SEQ ID NO:23.

107. The antibody of claim 29, in which the Delta protein is encoded by a first nucleic acid that hybridizes under low stringency conditions to a second nucleic acid, or its complement, which second nucleic acid is selected from the group consisting of the human *Delta* sequence of SEQ ID NO:14, the antisense strand to the human *Delta* sequence of SEQ ID NO:14, the human *Delta* sequence of SEQ ID NO:26, and the antisense strand to the human *Delta* sequence of SEQ ID NO:26, said low stringency conditions comprising pretreatment for 6 hours at 40°C in a solution containing 35% formamide, 5X SSC, 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 0.1% PVP, 0.1% Ficoll, 1% BSA, and 500 µg/ml denatured salmon sperm DNA; hybridization for 18-20 hours at 40°C in a solution containing 35% formamide, 5X SSC, 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 0.02% PVP, 0.02% Ficoll, 0.2% BSA, 100 µg/ml denatured salmon sperm DNA, and 10% (wt./vol.) dextran sulfate; washing for 1.5 hours at 55°C in a solution containing 2X SSC, 25 mM Tris-HCl (pH 7.4), 5 mM EDTA and 0.1% SDS; and a second washing for 1.5 hours at 60°C in a solution containing 2X SSC, 25 mM Tris-HCl (pH 7.4), 5 mM EDTA and 0.1% SDS.

108. The antibody of claim 29, in which the Delta protein is encoded by a first nucleic acid that hybridizes under low stringency conditions to a second nucleic acid, or its complement, which second nucleic acid is selected from the group consisting of the mouse-human *Delta* consensus sequence of SEQ ID NO:24, and the antisense strand to the mouse-human *Delta* consensus sequence of SEQ ID NO:24, said low stringency conditions comprising pretreatment for 6 hours at 40°C in a solution containing 35% formamide, 5X SSC, 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 0.1% PVP, 0.1% Ficoll, 1% BSA, and 500 µg/ml denatured salmon

sperm DNA; hybridization for 18-20 hours at 40°C in a solution containing 35% formamide, 5X SSC, 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 0.02% PVP, 0.02% Ficoll, 0.2% BSA, 100 µg/ml denatured salmon sperm DNA, and 10% (wt./vol.) dextran sulfate; washing for 1.5 hours at 55°C in a solution containing 2X SSC, 25 mM Tris-HCl (pH 7.4), 5 mM EDTA and 0.1% SDS; and a second washing for 1.5 hours at 60°C in a solution containing 2X SSC, 25 mM Tris-HCl (pH 7.4), 5 mM EDTA and 0.1% SDS.

109. A method of making an antibody comprising:

(a) administering an immunogenic amount of a vertebrate Delta protein to a host animal, in which the Delta protein is encoded by a first nucleic acid that hybridizes under low stringency conditions to a second nucleic acid, or its complement, which second nucleic acid is selected from the group consisting of the chick *Delta* sequence of SEQ ID NO:1, the antisense strand to the chick *Delta* sequence of SEQ ID NO:1, the chick *Delta* sequence of SEQ ID NO:3, the antisense strand to the chick *Delta* sequence of SEQ ID NO:3, the mouse *Delta* sequence of SEQ ID NO:11, the antisense strand to the mouse *Delta* sequence of SEQ ID NO:11, the human *Delta* sequence of SEQ ID NO:14, the antisense strand to the human *Delta* sequence of SEQ ID NO:14, the human *Delta* sequence of SEQ ID NO:26, the antisense strand to the human *Delta* sequence of SEQ ID NO:26, the mouse-human *Delta* consensus sequence of SEQ ID NO:24, and the antisense strand to the mouse-human *Delta* consensus sequence of SEQ ID NO:24, said low stringency conditions comprising pretreatment for 6 hours at 40°C in a solution containing 35% formamide, 5X SSC, 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 0.1% PVP, 0.1% Ficoll, 1% BSA, and 500 µg/ml denatured salmon sperm DNA; hybridization for 18-20 hours at 40°C in a solution containing 35% formamide, 5X SSC, 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 0.02% PVP, 0.02% Ficoll, 0.2% BSA, 100 µg/ml denatured salmon sperm DNA, and 10% (wt./vol.) dextran sulfate; washing for 1.5 hours at 55°C in a solution containing 2X SSC, 25 mM Tris-HCl (pH 7.4), 5 mM EDTA and 0.1% SDS; and a second washing for 1.5 hours at 60°C in a solution containing 2X SSC, 25 mM Tris-HCl (pH 7.4), 5 mM EDTA and 0.1% SDS, such that an antibody to said Delta protein is produced by said host animal; and

(b) recovering the antibody.

110. The method of claim 109, in which the Delta protein comprises the amino acid sequence of SEQ ID NOS:65-80.

111. The method of claim 109, in which the Delta protein comprises the amino acid sequence of SEQ ID NO:23.

112. The method of claim 109, in which the Delta protein comprises the amino acid sequence of SEQ ID NO:12.

113. The method of claim 109, in which the Delta protein is encoded by a first nucleic acid that hybridizes under low stringency conditions to a second nucleic acid, or its complement, which second nucleic acid is selected from the group consisting of the human *Delta* sequence of SEQ ID NO:14, the antisense strand to the human *Delta* sequence of SEQ ID NO:14, the human *Delta* sequence of SEQ ID NO:26, and the antisense strand to the human *Delta* sequence of SEQ ID NO:26, said low stringency conditions comprising pretreatment for 6 hours at 40°C in a solution containing 35% formamide, 5X SSC, 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 0.1% PVP, 0.1% Ficoll, 1% BSA, and 500 µg/ml denatured salmon sperm DNA; hybridization for 18-20 hours at 40°C in a solution containing 35% formamide, 5X SSC, 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 0.02% PVP, 0.02% Ficoll, 0.2% BSA, 100 µg/ml denatured salmon sperm DNA, and 10% (wt./vol.) dextran sulfate; washing for 1.5 hours at 55°C in a solution containing 2X SSC, 25 mM Tris-HCl (pH 7.4), 5 mM EDTA and 0.1% SDS; and a second washing for 1.5 hours at 60°C in a solution containing 2X SSC, 25 mM Tris-HCl (pH 7.4), 5 mM EDTA and 0.1% SDS.

114. A method of making an antibody comprising:

(a) administering an immunogenic amount of a fragment of a vertebrate Delta protein to a host animal, in which the fragment comprises a domain of the protein selected from the group consisting of the extracellular domain, DSL domain, domain amino-terminal to the DSL domain, epidermal growth factor-like repeat domain, transmembrane domain, and intracellular domain, in which the Delta protein is comprises an amino acid sequence encoded by a first nucleic acid that hybridizes under low stringency conditions to a second nucleic acid, or its complement, which second nucleic acid is selected from the group consisting of the chick *Delta* sequence of SEQ ID NO:1, the antisense strand to the chick *Delta* sequence of SEQ ID NO:1, the chick *Delta* sequence of SEQ ID NO:3, the antisense strand to the chick *Delta* sequence of SEQ ID NO:3, the mouse *Delta* sequence of SEQ ID NO:11, the antisense strand to the mouse *Delta* sequence of SEQ ID NO:11, the human *Delta* sequence of SEQ ID NO:14, the antisense strand to the human

Delta sequence of SEQ ID NO:14, the human *Delta* sequence of SEQ ID NO:26, the antisense strand to the human *Delta* sequence of SEQ ID NO:26, the mouse-human *Delta* consensus sequence of SEQ ID NO:24, and the antisense strand to the mouse-human *Delta* consensus sequence of SEQ ID NO:24, said low stringency conditions comprising pretreatment for 6 hours at 40°C in a solution containing 35% formamide, 5X SSC, 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 0.1% PVP, 0.1% Ficoll, 1% BSA, and 500 µg/ml denatured salmon sperm DNA; hybridization for 18-20 hours at 40°C in a solution containing 35% formamide, 5X SSC, 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 0.02% PVP, 0.02% Ficoll, 0.2% BSA, 100 µg/ml denatured salmon sperm DNA, and 10% (wt./vol.) dextran sulfate; washing for 1.5 hours at 55°C in a solution containing 2X SSC, 25 mM Tris-HCl (pH 7.4), 5 mM EDTA and 0.1% SDS; and a second washing for 1.5 hours at 60°C in a solution containing 2X SSC, 25 mM Tris-HCl (pH 7.4), 5 mM EDTA and 0.1% SDS, such that an antibody to said fragment is produced by said host animal; and

(b) recovering the antibody.

115. The method of claim 114, in which the fragment of the vertebrate Delta protein comprises the membrane-associated region of the Delta protein.

116. The method of claim 114, in which the fragment of the vertebrate Delta protein comprises an epidermal growth factor-homologous repeat of the protein.

117. The method of claim 114, in which the fragment of the vertebrate Delta protein consists of at least 20 contiguous amino acids of the vertebrate Delta protein.

118. The method of claim 114, in which the fragment of the vertebrate Delta protein lacks the transmembrane and intracellular domain of the protein.

119. The method of claim 114, in which the fragment of the vertebrate Delta protein lacks the extracellular domain of the protein.

120. The method of claim 114, in which the fragment of the vertebrate Delta protein lacks the epidermal growth factor-like repeats of the protein.

121. An antibody produced by the method of claim 109, which does not bind a *Drosophila* Delta protein.

122. An antibody produced by the method of claim 114, which does not bind a *Drosophila* Delta protein.

123. The antibody of claim 121 or 122, in which the antibody is monoclonal.

124. The antibody of claim 121, 122 or 123, in which the antibody is purified.

125. A composition comprising an amount of an antibody of claim 121, 122, 123 or 124, and a pharmaceutically acceptable carrier.

126. The method of claim 109 or 114, in which the Delta protein comprises an amino acid sequence selected from the group consisting of the chick Delta sequence of SEQ ID NO:2, the mouse Delta sequence of SEQ ID NO:12, the human Delta sequence of SEQ ID NO:23, and the human Delta sequence of SEQ ID NOS:65-80.

127. The method of claim 109 or 114, in which the Delta protein comprises an amino acid sequence selected from the group consisting of the human Delta sequence of SEQ ID NO:23.

128. The method of claim 109 or 114, in which the Delta protein comprises an amino acid sequence selected from the group consisting of the human Delta sequence of SEQ ID NOS:65-80.

129. A method of making an antibody comprising:

(a) administering an immunogenic amount of a protein comprising a fragment of a vertebrate Delta protein to a host animal, in which the fragment comprises a domain of the protein selected from the group consisting of the extracellular domain, DSL domain, domain amino-terminal to the DSL domain, epidermal growth factor-like repeat domain, transmembrane domain, and intracellular domain, in which the Delta protein is comprises an amino acid sequence encoded by a first nucleic acid that hybridizes under low stringency conditions to a second nucleic acid, or its complement, which second nucleic acid is selected from the group consisting of the chick *Delta* sequence of SEQ ID NO:1, the antisense strand to the chick *Delta* sequence of SEQ ID NO:1, the chick *Delta* sequence of SEQ ID NO:3, the antisense strand to the chick *Delta* sequence of SEQ ID NO:3, the mouse *Delta* sequence of SEQ ID NO:11, the antisense strand to the mouse *Delta* sequence of SEQ ID NO:11, the human *Delta* sequence of SEQ ID NO:14, the antisense strand to the human *Delta* sequence of SEQ ID NO:14, the human *Delta* sequence of SEQ ID NO:26, the antisense strand to the human *Delta* sequence of SEQ ID NO:26, the mouse-human *Delta* consensus sequence of SEQ ID NO:24, and the antisense strand to the mouse-human *Delta* consensus sequence of SEQ ID NO:24, said low stringency

conditions comprising pretreatment for 6 hours at 40°C in a solution containing 35% formamide, 5X SSC, 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 0.1% PVP, 0.1% Ficoll, 1% BSA, and 500 µg/ml denatured salmon sperm DNA; hybridization for 18-20 hours at 40°C in a solution containing 35% formamide, 5X SSC, 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 0.02% PVP, 0.02% Ficoll, 0.2% BSA, 100 µg/ml denatured salmon sperm DNA, and 10% (wt./vol.) dextran sulfate; washing for 1.5 hours at 55°C in a solution containing 2X SSC, 25 mM Tris-HCl (pH 7.4), 5 mM EDTA and 0.1% SDS; and a second washing for 1.5 hours at 60°C in a solution containing 2X SSC, 25 mM Tris-HCl (pH 7.4), 5 mM EDTA and 0.1% SDS, such that an antibody to said Delta fragment is produced by said host animal; and

(b) recovering the antibody.

130. The method according to claim 129, in which the fragment of the Delta protein is joined via a peptide bond to an amino acid sequence of a second protein, in which the second protein is not the Delta protein.

131. A method of making a monoclonal antibody comprising:

(a) administering an immunogenic amount of a vertebrate Delta protein to a mouse, in which the Delta protein is encoded by a first nucleic acid that hybridizes under low stringency conditions to a second nucleic acid, or its complement, which second nucleic acid is selected from the group consisting of the chick *Delta* sequence of SEQ ID NO:1, the antisense strand to the chick *Delta* sequence of SEQ ID NO:1, the chick *Delta* sequence of SEQ ID NO:3, the antisense strand to the chick *Delta* sequence of SEQ ID NO:3, the mouse *Delta* sequence of SEQ ID NO:11, the antisense strand to the mouse *Delta* sequence of SEQ ID NO:11, the human *Delta* sequence of SEQ ID NO:14, the antisense strand to the human *Delta* sequence of SEQ ID NO:14, the human *Delta* sequence of SEQ ID NO:26, the antisense strand to the human *Delta* sequence of SEQ ID NO:26, the mouse-human *Delta* consensus sequence of SEQ ID NO:24, and the antisense strand to the mouse-human *Delta* consensus sequence of SEQ ID NO:24, said low stringency conditions comprising pretreatment for 6 hours at 40°C in a solution containing 35% formamide, 5X SSC, 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 0.1% PVP, 0.1% Ficoll, 1% BSA, and 500 µg/ml denatured salmon sperm DNA; hybridization for 18-20 hours at 40°C in a solution containing 35% formamide, 5X SSC, 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 0.02% PVP, 0.02% Ficoll, 0.2% BSA, 100 µg/ml denatured salmon sperm DNA, and 10% (wt./vol.)

dextran sulfate; washing for 1.5 hours at 55°C in a solution containing 2X SSC, 25 mM Tris-HCl (pH 7.4), 5 mM EDTA and 0.1% SDS; and a second washing for 1.5 hours at 60°C in a solution containing 2X SSC, 25 mM Tris-HCl (pH 7.4), 5 mM EDTA and 0.1% SDS;

(b) recovering spleen cells from said mouse;

(c) fusing the recovered spleen cells with a cell of a mouse myeloma to generate hybridomas;

(d) screening to select a hybridoma producing antibody to said vertebrate Delta protein; and

(e) recovering the antibody.

132. A method of making a monoclonal antibody comprising:

(a) fusing a spleen cell from a mouse immunized with an immunogenic amount of a vertebrate Delta protein with a cell of a mouse myeloma to generate hybridomas, in which the Delta protein is encoded by a first nucleic acid that hybridizes under low stringency conditions to a second nucleic acid, or its complement, which second nucleic acid is selected from the group consisting of the chick *Delta* sequence of SEQ ID NO:1, the antisense strand to the chick *Delta* sequence of SEQ ID NO:1, the chick *Delta* sequence of SEQ ID NO:3, the antisense strand to the chick *Delta* sequence of SEQ ID NO:3, the mouse *Delta* sequence of SEQ ID NO:11, the antisense strand to the mouse *Delta* sequence of SEQ ID NO:11, the human *Delta* sequence of SEQ ID NO:14, the antisense strand to the human *Delta* sequence of SEQ ID NO:14, the human *Delta* sequence of SEQ ID NO:26, the antisense strand to the human *Delta* sequence of SEQ ID NO:26, the mouse-human *Delta* consensus sequence of SEQ ID NO:24, and the antisense strand to the mouse-human *Delta* consensus sequence of SEQ ID NO:24, said low stringency conditions comprising pretreatment for 6 hours at 40°C in a solution containing 35% formamide, 5X SSC, 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 0.1% PVP, 0.1% Ficoll, 1% BSA, and 500 µg/ml denatured salmon sperm DNA; hybridization for 18-20 hours at 40°C in a solution containing 35% formamide, 5X SSC, 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 0.02% PVP, 0.02% Ficoll, 0.2% BSA, 100 µg/ml denatured salmon sperm DNA, and 10% (wt./vol.) dextran sulfate; washing for 1.5 hours at 55°C in a solution containing 2X SSC, 25 mM Tris-HCl (pH 7.4), 5 mM EDTA and 0.1% SDS; and a second washing for 1.5 hours at 60°C in a solution containing 2X SSC, 25 mM Tris-HCl (pH 7.4), 5 mM EDTA and 0.1% SDS;

(b) screening to select a hybridoma producing antibody to said vertebrate Delta protein; and

(c) recovering the antibody.

133. A method of making a monoclonal antibody comprising:

(a) administering an immunogenic amount of a vertebrate Delta protein to a host animal, in which the Delta protein is encoded by a first nucleic acid that hybridizes under low stringency conditions to a second nucleic acid, or its complement, which second nucleic acid is selected from the group consisting of the chick *Delta* sequence of SEQ ID NO:1, the antisense strand to the chick *Delta* sequence of SEQ ID NO:1, the chick *Delta* sequence of SEQ ID NO:3, the antisense strand to the chick *Delta* sequence of SEQ ID NO:3, the mouse *Delta* sequence of SEQ ID NO:11, the antisense strand to the mouse *Delta* sequence of SEQ ID NO:11, the human *Delta* sequence of SEQ ID NO:14, the antisense strand to the human *Delta* sequence of SEQ ID NO:14, the human *Delta* sequence of SEQ ID NO:26, the antisense strand to the human *Delta* sequence of SEQ ID NO:26, the mouse-human *Delta* consensus sequence of SEQ ID NO:24, and the antisense strand to the mouse-human *Delta* consensus sequence of SEQ ID NO:24, said low stringency conditions comprising pretreatment for 6 hours at 40°C in a solution containing 35% formamide, 5X SSC, 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 0.1% PVP, 0.1% Ficoll, 1% BSA, and 500 µg/ml denatured salmon sperm DNA; hybridization for 18-20 hours at 40°C in a solution containing 35% formamide, 5X SSC, 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 0.02% PVP, 0.02% Ficoll, 0.2% BSA, 100 µg/ml denatured salmon sperm DNA, and 10% (wt./vol.) dextran sulfate; washing for 1.5 hours at 55°C in a solution containing 2X SSC, 25 mM Tris-HCl (pH 7.4), 5 mM EDTA and 0.1% SDS; and a second washing for 1.5 hours at 60°C in a solution containing 2X SSC, 25 mM Tris-HCl (pH 7.4), 5 mM EDTA and 0.1% SDS;

(b) recovering lymphocytes from said host animal;

(c) fusing the recovered lymphocytes with a cell of a myeloma, plastocytoma or lymphoblastoid cell line to generate hybridomas;

(d) screening to select a hybridoma producing antibody to said vertebrate Delta protein; and

(e) recovering the antibody.

134. A method of making a monoclonal antibody comprising:

(a) fusing a lymphocyte from a host animal immunized with an immunogenic amount of a vertebrate Delta protein with a cell of a myeloma, plastocytoma or lymphoblastoid cell line to generate hybridomas, in which the Delta protein is encoded by a first nucleic acid that hybridizes under low stringency conditions to a second nucleic acid, or its complement, which second nucleic acid is selected from the group consisting of the chick *Delta* sequence of SEQ ID NO:1, the antisense strand to the chick *Delta* sequence of SEQ ID NO:1, the chick *Delta* sequence of SEQ ID NO:3, the antisense strand to the chick *Delta* sequence of SEQ ID NO:3, the mouse *Delta* sequence of SEQ ID NO:11, the antisense strand to the mouse *Delta* sequence of SEQ ID NO:11, the human *Delta* sequence of SEQ ID NO:14, the antisense strand to the human *Delta* sequence of SEQ ID NO:14, the human *Delta* sequence of SEQ ID NO:26, the antisense strand to the human *Delta* sequence of SEQ ID NO:26, the mouse-human *Delta* consensus sequence of SEQ ID NO:24, and the antisense strand to the mouse-human *Delta* consensus sequence of SEQ ID NO:24, said low stringency conditions comprising pretreatment for 6 hours at 40°C in a solution containing 35% formamide, 5X SSC, 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 0.1% PVP, 0.1% Ficoll, 1% BSA, and 500 µg/ml denatured salmon sperm DNA; hybridization for 18-20 hours at 40°C in a solution containing 35% formamide, 5X SSC, 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 0.02% PVP, 0.02% Ficoll, 0.2% BSA, 100 µg/ml denatured salmon sperm DNA, and 10% (wt./vol.) dextran sulfate; washing for 1.5 hours at 55°C in a solution containing 2X SSC, 25 mM Tris-HCl (pH 7.4), 5 mM EDTA and 0.1% SDS; and a second washing for 1.5 hours at 60°C in a solution containing 2X SSC, 25 mM Tris-HCl (pH 7.4), 5 mM EDTA and 0.1% SDS;

(b) screening to select a hybridoma producing antibody to said vertebrate Delta protein; and

(c) recovering the antibody.

135. A method of making a monoclonal antibody comprising:

(a) administering an immunogenic amount of a vertebrate Delta protein to a host animal, in which the Delta protein is encoded by a first nucleic acid that hybridizes under low stringency conditions to a second nucleic acid, or its complement, which second nucleic acid is selected from the group consisting of the chick *Delta* sequence of SEQ ID NO:1, the antisense strand to the chick *Delta* sequence of SEQ ID NO:1, the chick *Delta* sequence of SEQ ID NO:3, the

antisense strand to the chick *Delta* sequence of SEQ ID NO:3, the mouse *Delta* sequence of SEQ ID NO:11, the antisense strand to the mouse *Delta* sequence of SEQ ID NO:11, the human *Delta* sequence of SEQ ID NO:14, the antisense strand to the human *Delta* sequence of SEQ ID NO:14, the human *Delta* sequence of SEQ ID NO:26, the antisense strand to the human *Delta* sequence of SEQ ID NO:26, the mouse-human *Delta* consensus sequence of SEQ ID NO:24, and the antisense strand to the mouse-human *Delta* consensus sequence of SEQ ID NO:24, said low stringency conditions comprising pretreatment for 6 hours at 40°C in a solution containing 35% formamide, 5X SSC, 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 0.1% PVP, 0.1% Ficoll, 1% BSA, and 500 μg/ml denatured salmon sperm DNA; hybridization for 18-20 hours at 40°C in a solution containing 35% formamide, 5X SSC, 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 0.02% PVP, 0.02% Ficoll, 0.2% BSA, 100 μg/ml denatured salmon sperm DNA, and 10% (wt./vol.) dextran sulfate; washing for 1.5 hours at 55°C in a solution containing 2X SSC, 25 mM Tris-HCl (pH 7.4), 5 mM EDTA and 0.1% SDS; and a second washing for 1.5 hours at 60°C in a solution containing 2X SSC, 25 mM Tris-HCl (pH 7.4), 5 mM EDTA and 0.1% SDS;

(b) recovering lymphocytes from said host animal;

(c) immortalizing the recovered lymphocytes with Epstein-Barr virus to generate immortalized cells;

(d) screening to select an immortalized cell producing antibody to said vertebrate Delta protein; and

(e) recovering the antibody.

136. A method of making a monoclonal antibody comprising:

(a) immortalizing a lymphocyte from a host animal immunized with an immunogenic amount of a vertebrate Delta protein with Epstein-Barr virus to generate immortalized cells, in which the Delta protein is encoded by a first nucleic acid that hybridizes under low stringency conditions to a second nucleic acid, or its complement, which second nucleic acid is selected from the group consisting of the chick *Delta* sequence of SEQ ID NO:1, the antisense strand to the chick *Delta* sequence of SEQ ID NO:1, the chick *Delta* sequence of SEQ ID NO:3, the antisense strand to the chick *Delta* sequence of SEQ ID NO:3, the mouse *Delta* sequence of SEQ ID NO:11, the antisense strand to the mouse *Delta* sequence of SEQ ID NO:11, the human *Delta* sequence of SEQ ID NO:14, the antisense strand to the human *Delta* sequence of SEQ ID

NO:14, the human *Delta* sequence of SEQ ID NO:26, the antisense strand to the human *Delta* sequence of SEQ ID NO:26, the mouse-human *Delta* consensus sequence of SEQ ID NO:24, and the antisense strand to the mouse-human *Delta* consensus sequence of SEQ ID NO:24, said low stringency conditions comprising pretreatment for 6 hours at 40°C in a solution containing 35% formamide, 5X SSC, 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 0.1% PVP, 0.1% Ficoll, 1% BSA, and 500 μg/ml denatured salmon sperm DNA; hybridization for 18-20 hours at 40°C in a solution containing 35% formamide, 5X SSC, 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 0.02% PVP, 0.02% Ficoll, 0.2% BSA, 100 μg/ml denatured salmon sperm DNA, and 10% (wt./vol.) dextran sulfate; washing for 1.5 hours at 55°C in a solution containing 2X SSC, 25 mM Tris-HCl (pH 7.4), 5 mM EDTA and 0.1% SDS; and a second washing for 1.5 hours at 60°C in a solution containing 2X SSC, 25 mM Tris-HCl (pH 7.4), 5 mM EDTA and 0.1% SDS;

(b) screening to select an immortalized cell producing antibody to said vertebrate Delta protein; and

(c) recovering the antibody.

137. A method of producing a phage Fab expression library comprising:

(a) isolating spleen cells from a host animal immunized with an immunogenic amount of a vertebrate Delta protein, in which the Delta protein is encoded by a first nucleic acid that hybridizes under low stringency conditions to a second nucleic acid, or its complement, which second nucleic acid is selected from the group consisting of the chick *Delta* sequence of SEQ ID NO:1, the antisense strand to the chick *Delta* sequence of SEQ ID NO:1, the chick *Delta* sequence of SEQ ID NO:3, the antisense strand to the chick *Delta* sequence of SEQ ID NO:3, the mouse *Delta* sequence of SEQ ID NO:11, the antisense strand to the mouse *Delta* sequence of SEQ ID NO:11, the human *Delta* sequence of SEQ ID NO:14, the antisense strand to the human *Delta* sequence of SEQ ID NO:14, the human *Delta* sequence of SEQ ID NO:26, the antisense strand to the human *Delta* sequence of SEQ ID NO:26, the mouse-human *Delta* consensus sequence of SEQ ID NO:24, and the antisense strand to the mouse-human *Delta* consensus sequence of SEQ ID NO:24, said low stringency conditions comprising pretreatment for 6 hours at 40°C in a solution containing 35% formamide, 5X SSC, 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 0.1% PVP, 0.1% Ficoll, 1% BSA, and 500 μg/ml denatured salmon sperm DNA; hybridization for 18-20 hours at 40°C in a solution containing 35% formamide, 5X SSC, 50 mM

Tris-HCl (pH 7.5), 5 mM EDTA, 0.02% PVP, 0.02% Ficoll, 0.2% BSA, 100 μg/ml denatured salmon sperm DNA, and 10% (wt./vol.) dextran sulfate; washing for 1.5 hours at 55°C in a solution containing 2X SSC, 25 mM Tris-HCl (pH 7.4), 5 mM EDTA and 0.1% SDS; and a second washing for 1.5 hours at 60°C in a solution containing 2X SSC, 25 mM Tris-HCl (pH 7.4), 5 mM EDTA and 0.1% SDS;

(b) amplifying, by polymerase chain reaction, antibody heavy and light chain nucleotide sequences from messenger RNA isolated from the spleen cells;

(c) cloning the amplified heavy chain and light chain nucleotide sequences into a lambda phage vector to produce a heavy chain library and a light chain library, respectively;

(d) combining and ligating the heavy and light chain nucleotide sequences from the heavy chain and light chain libraries to produce a phage Fab expression library that co-expresses antibody heavy and light chains; and

(e) screening the expression library for a phage that binds said Delta protein.

138. The method of claim 131, 132, 133, 134, 135, 136 or 137, which second nucleic acid is selected from the group consisting of the human *Delta* sequence of SEQ ID NO:14, the antisense strand to the human *Delta* sequence of SEQ ID NO:14, the human *Delta* sequence of SEQ ID NO:26, the antisense strand to the human *Delta* sequence of SEQ ID NO:26, the mouse-human *Delta* consensus sequence of SEQ ID NO:24, and the antisense strand to the mouse-human *Delta* consensus sequence of SEQ ID NO:24.

139. The method of claim 131, 132, 133, 134, 135, 136 or 137, in which the Delta protein comprises the amino acid sequence of SEQ ID NOS:65-80.

140. The method of claim 131, 132, 133, 134, 135, 136 or 137, in which the Delta protein comprises the amino acid sequence of SEQ ID NO:23.

141. The method of claim 131, 132, 133, 134, 135, 136 or 137, in which the Delta protein comprises the amino acid sequence of SEQ ID NO:12.

142. An antibody produced by the method of claim 131, 132, 133, 134, 135, 136 or 137, which does not bind a *Drosophila* Delta protein.

143. The antibody of claim 142, in which the antibody is purified.

144. A composition comprising the antibody of claim 142, and a pharmaceutically acceptable carrier.

145. A composition comprising the antibody of claim 143, and a pharmaceutically acceptable carrier.

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The production of monoclonal antibodies from human lymphocytes

Danuta Kozbor and John C. Roder

Two conceptually unique approaches are currently available for the production of human monoclonal antibodies – the 'hybridoma' technique, based on the fusion of antibody-producing B lymphocytes with plasmacytoma cells or lymphoblastoid cell lines; and the use of Epstein-Barr virus (EBV) to 'immortalize' antigen-specific, human B lymphocytes. Here Danuta Kozbor and John Roder discuss the contribution each technique is making, alone or in combination, towards the desired aim of producing human monoclonal antibodies with a defined specificity.

The hybridoma technique

Murine plasmacytomas as fusion partners

The production of human monoclonal antibodies by fusion[1] has been hampered, mainly by the current scarcity of human cell lines which when fused will support the secretion of immunoglobulin. In the meantime, HAT-sensitive murine plasmacytomas have been fused with human lymphocytes, to yield mouse × human hybrids that secrete human antibody against the Forssman antigen[2], human mammary carcinoma cells[3], keyhole limpet hemocyanin (KLH)[4] and tetanus toxoid[5]. These interspecies hybridomas preferentially segregate human chromosomes, making the derivation of stable lines secreting human antibody an enormously laborious task. However, loss of human chromosomes from mouse × human hybridomas is not random. It is known that human chromosomes 14 (heavy chain) and 22 (light chain-λ) are preferentially retained, whereas chromosomes 2 (light chain-κ) is preferentially lost[6,7]. Even hybrids possessing the appropriate human chromosomes often fail to secrete human Ig, because the appropriate environmental triggering stimuli are absent[8].

Human plasmacytomas as fusion partners

Since the chromosomal constitution of intraspecific human hybrids is much more stable, human × human hybridomas are more likely to be a useful source of specific human monoclonal antibodies. However, cells of the most differentiated human lymphoid neoplasia, the plasmacytoma, can only rarely be maintained in continuous culture. The basis for classification of these lines lies in the identity between the myeloma protein *in vivo* and the immunoglobulin synthesized *in vitro*. In addition, plasmacytoma cells have abundant rough endoplasmic reticulum (RER), few free polyribosomes, numerous mitochondria, and a well-developed Golgi apparatus. These cells never carry Epstein-Barr virus (EBV) and are usually aneuploid[9]. Despite numerous efforts to establish long-term cultures of human plasmacytomas, only 10 lines which fulfil the above criteria have been reported to date; as shown in Table I[10–18]. Of these only 2 have been rendered deficient in the purine salvage enzyme, hypoxanthine-guanine phosphoribosyl transferase (HGPRT) and are suitable as fusion partners and for selection in medium containing hypoxanthine, aminopterin and thymidine (HAT). (Table II).

The first reported human hybridoma produced monoclonal antibodies against 2,4-dinitrophenyl (DNP) hapten and was obtained with the HAT-sensitive plasma cell line U-266AR1 which was renamed SK0-007 (Ref. 19) and which originated from the IgE-producing myeloma U-266. It was reported elsewhere that among HGPRT⁻ sublines of U-266, ease of fusion correlated with rate of division[20] and that U-266AR1 divided twice as fast as the U-266 parental line[11]. This subline U-266AR1 was fused with the uninvolved splenic lymphocytes from a Hodg-

TABLE I. Characterization of human myeloma cell lines

Cell line	Tissue of origin	Karyotype	Class of Ig produced *In vivo*	Class of Ig produced *In vitro*	Ref.
RPMI 8226	Peripheral blood	Near triploid	γ,λ	λ	10
ARH 77[a]	Peripheral blood	45–56	γ,λ	γ,λ	14
L 363	Peripheral blood	49, X, +8M, −5, −6, +7, −8, 14q+, −22	γ	Not detected	15
266 BL	Peripheral blood	Near diploid	ε,λ	ε,λ	11
268 BM	Bone marrow	Near diploid	ε,λ	ε,λ	11
LA 49	Pleural effusion	Polyploidy (23–250)	δ,λ	δ,λ	12
Oda	Subcutaneous plasmacytoma	46	δ,λ	δ,λ	13
KMM 56	Pleural effusion	71 (49–144)	λ	λ	16
Karpas 707	Peripheral blood and bone marrow	45, XY, Ph+	γ,λ	λ	17
KMM – 1	Subcutaneous plasmacytoma	47, X, −Y, 1q+, −2, +t (1:2) (cen:cen), +7, 12q+, 14q+, +mar	λ	λ	18

[a]ARH 77 appears to be a LCL since it was subsequently found to be EBNA-positive[32].

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kin's lymphoma patient sensitized with dinitrochlorobenzene over 2 weeks prior to surgery. In experiments with 3 separate spleens, average fusion frequencies of 37×10^{-7} were calculated from the data given. Approximately 28% of wells containing hybrids produced IgG and 1.7% of all hybrids were specific for DNP. Hybrids produced 3–11 $\mu g\ ml^{-1}\ day^{-1}$ of monoclonal IgG, anti-DNP antibodies. Attempts to reproduce the results were not successful for two years, probably due to a mycoplasma infection of the cells[21]. Apparently a mycoplasma-free subline of SK0-007 has been used successfully by Cote at the Sloan-Kettering Institute, New York (reported by Sikora and Neville, Ref. 22).

Another HAT-sensitive fusion partner of putative plasmacytoma origin, TM-H2, was derived from the RPMI-8226 line[23]. However, unlike the parental line which secretes λ light-chain dimers[10], TM-H2 secretes IgGκ. Because it is unlikely that there are genetic changes in both the heavy-and light-chain genes, and in view of two other reports that some RPMI-8226 lines in circulation are of mouse[24] and non-human primate origin[25], one would like to see definitive karyotypic and isoenzymatic evidence that TM-H2 is of human origin. Lymphocytes from the peripheral blood or bone marrow of normal donors were fused with TM-H2 and 1.3–7.4% of all hybrids produced natural antibodies against sheep erythrocytes (SRBC) or various human tumor cell lines[23]. Hybridization frequencies were remarkably high (10^{-1}) (G. Price, personal communication), and putative hybridomas secreted up to 10 μg antibody ml^{-1} $(10^6\ cells)^{-1}$. Most reactions were rather weak and in the absence of titrations we assume that these natural monoclonal antibodies are of low affinity. Stability was not mentioned and no formal proof was presented that the authors were dealing with true hybrids rather than lymphoblastoid cell lines which might have arisen from the donor lymphocyte population.

TABLE II. Human fusion partners

Fusion Partner					Hybridomas								
Fusion partner	Cell type	Class of Ig secreted	Resistant to	Source of donor lymphocytes[c]	Fusion frequency ($\times 10^{-7}$)	Specific hybrids (%)	Specificity	Ig secretion (μg/ml)	Specific Ig class	Cloning efficiency (%)	Division time (h)	Stability (months)	Availability of fusion partner[e]	Ref.
SKO-007	Plasmacytoma	ε,λ	8-AG	Immune spleen (Hodgkin's)	37	1.7	DNP hapten	3–11	IgG	ND[b]	ND	ND	Yes	19
TM-H2	Plasma-cytoma[d]	λ,κ	6-Thg	Normal PBL, BM	ND	1.3–7.4	SRBC HL-60	10	ND	ND	ND	ND	No	23
DHMC	Plasmacytoma	γ,λ	6-Thg	CLL PBL, spleen	(10–100)[g]	ND	SRBC	< 20	IgM	ND	53[f]	> 3	No	26
GM1500	LCL[h]	γ2,κ	6-Thg	Immune PBL (SSPE)	18	ND	Measles virus	ND	IgM	ND	ND	ND	Yes	27
				Type I-diabetes, PBL	25	4.5	Islet cells	0.4	IgM	ND	ND	> 12	Yes	30
KR-4	LCL	γ,κ	6-Thg, Oua	TT-specific EBV line	112	94	Tetanus toxoid	6	IgM	64	24	> 12	Yes	31
				Immune PBL, (EBV)	36	0.7–4	Tetanus toxoid	3.5	IgM	70	22	> 6	Yes	48
LICR-LON-HMy2	LCL	γ1,κ	8-AG	Normal PBL, tonsil lymph-node	0.1–10	None	None	0.5–8	IgG	25	26	> 12	Yes	32
				TIL	ND	10	Glioma cells	ND	ND	ND	27	ND	Yes	33
GM0467.3	LCL	μ,λ	8-AG	Immune tonsil (PWM)	22	4.7	Tetanus toxoid	0.4–2.8	IgM	High	ND	> 9	?	34
GM351.1	LCL	μ,κ	8-AG	Normal PBL, spleen (PWM)	22	None	None	ND	ND	High	ND	ND	?	34
UC729-6	LCL	μ,κ	6-Thg	Lymph-node (cancer patient)	ND	ND	Carcinoma lines	3–10	IgM	ND	ND	ND	?	36
GM4672	LCL	γ2,κ	6-Thg	Immune PBL	ND	ND	Rh(D)	0.1–1.0	IgG	ND	ND	ND	?	37
				Auto immune PBL, spleen	17	15	ssDNA, platelets, erythro-cytes	1–15	IgM	ND	ND	> 7	?	38

[a] Cloning efficiency determined by limiting dilution.
[b] ND: not done.
[c] PBL: peripheral blood lymphocytes; BM: bone marrow; CLL: chronic lymphocytic leukemia; SSPE: subacute sclerozing panencephalitis; TT: tetanus toxoid; EBV: Epstein-Barr virus; TIL: tumor infiltrating lymphocytes; PWM: pokeweed mitogen.
[d] TM-H2 derives from RPM18226 which is a plasmacytoma. However, TM-H2 secretes γ,κ rather than λ light-chain dimers and therefore cannot be assumed to be a plasmacytoma, at present.
[e] The fusion partner was requested from each author and 6 months given to send the line. In other cases, the authors stated at meetings that the line was not available. Exchange of the line between non-collaborating laboratories was also taken as evidence of general availability. KR-4 has been sent out to all three laboratories who have requested it to date.
[f] Division time for the fusion partner. Data on hybridoma not available.
[g] Estimate based on assumptions made concerning the number of cells seeded per well.
[h] LCL: lymphoblastoid cell line.

Preliminary reports suggest that DHMC may also be a suitable plasmacytoma fusion partner[26]. This line was EBNA$^-$, mycoplasma-free, cytoplasmic Ig$^+$, and possessed abundant RER; it had 46–47 chromosomes; divided every 53 h; and secreted low levels of Ig. Hybridization between cultured chromic lymphocytic leukemia (CLL) cells specific for SRBC and DHMC yielded 2.5–84 hybrids/10^7 donor cells. These hybrids secreted up to 20 μg/ml IgMλ over the 3 months of the study but it is not clear how the authors selected against the CLL line which also secreted IgM of the same anti-SRBC specificity.

Human lymphoblastoid cell lines as fusion partners

The paucity of human myeloma lines has prompted other investigators to construct human × human hybrids with lymphoblastoid cell lines (LCLs). LCLs established from malignant or normal hematopoietic tissue show constant association with EBV, polyclonal derivation, and diploidy. They have numerous free polyribosomes and a poorly developed RER and Golgi apparatus. Some of these phenotypic features are characteristic for certain stages in B-cell differentiation and may play a crucial role in supporting the production of antibodies when fused with normal B cells. The first LCL fusion partner was made by Croce *et al.*[27] in 1980 and originated from a B-cell line, GM1500, established from a patient with multiple myeloma. This line, GM1500 6TG-2, secreted IgG2κ and was reported by Kozbor *et al.*[28] to bear the EBV-induced nuclear antigen, EBNA, as shown previously for the parental GM1500 line[29]. Peripheral blood mononuclear cells were obtained from a lethally infected patient with subacute sclerosing panencephalitis who had extremely high titers (10^{-6}) of anti-measles antibody in her serum. These were fused with GM1500 6TG-2 and six clones secreting IgM specific for measles virus nucleocapsids were isolated. Hybridization frequencies of 18 × 10^{-7} were estimated from data given in the paper and although it was not possible to calculate the percentage of hybrids specific for the measles virus antigen, the number is probably high. This same fusion partner, GM1500 6TG-2, was fused in a later study with peripheral blood mononuclear cells from a patient with type-I diabetes mellitus of 5 months' duration[30]. Only 1 out of 3 attempted fusions was successful and in this case a hybridization frequency of 25 × 10^{-7} was obtained. One clone (4.5% of all hybrids) produced IgM specific for islet cells in the pancreas. This clone has been stable for over a year and secretes approximately 0.4 μg IgM/ml.

The GM1500 6TG-2 cell line has also been mutagenized and rendered ouabain-resistant[31]. The resulting fusion partner, called KR-4 was hybridized with EBV-transformed cell lines specific for tetanus toxoid. Hybridization frequencies as high as 112 × 10^{-7} were obtained and 96% of all hybrids stably secreted IgM specific for tetanus toxoid at levels as high as 6 μg/ml for over 1 year to the present time. Much lower hybridization frequencies (4 × 10^{-7}) were obtained between KR-4 and fresh, non-transformed peripheral blood lymphocytes.

Another LCL fusion partner, LICR-LON-HMy2, was derived from ARH 77. This line is EBNA$^+$, hypodiploid (43 chromosomes) and secretes IgG1κ (Ref. 32). It has a doubling time of 20 h and a cloning efficiency of 25% by limiting dilution. The line is mycoplasma-free by the fluorescent staining test and possesses human isoenzymes. Lymphocytes from peripheral blood, tonsil or lymph node all fused with frequencies of approximately 1 × 10^{-7}. Hybrids had a model chromosome number of 60 (55–85) and secreted 0.5–8 μg/ml of IgG. Other investigators have successfully derived glioma-specific hybridomas by fusing LICR-LON-HMy2 with tumor-infiltrating lymphocytes[33]. In a large study of 180 tumor patients, cells from draining lymph nodes were successfully hybridized in 24 cases and in 9 of these hybridomas were obtained which secreted antibody with weak binding to tumor cells. A third laboratory (Cote) has also reported success with the LICR-LON-HMy2 line[22].

Chiorazzi *et al.* cloned H351.1 (IgMκ) from the parental line WI-L2 AG^{R}35sc1, and 0467.3 (IgMλ) from the parental line PGLC33H (Ref. 34). These clones hybridized equally well at estimated frequencies of 22 × 10^{-7} with lymphocytes from spleen, tonsil or peripheral blood stimulated *in vitro* with pokeweed mitogen (PWM). Most (89%) of the hybrids secreted IgM whereas few (11%) secreted IgG. In one case tonsil lymphocytes from a donor immunized with tetanus toxoid (TT) 1 week before surgery was fused with 0467.3. Of 21 hybrids (4.7%) 1 secreted anti-TT and subclones secreted levels of 0.4–2.8 μg/ml for the duration of the study (9 months). A separate group has also reported success in deriving IgA, IgG and IgM monoclonal antibody against sheep erythrocytes by fusing WI-L2-729HF2 with tonsil lymphocytes immunized *in vitro*[35].

Another LCL, UC729-6, has been fused with the draining lymph node cells of cancer patients and two hybrids were obtained secreting 3–10 μg/ml of IgM or IgG antibody which reacted with human tumor cell lines but not normal cells[36]. The UC729-6 line is a non-Ig secretor, cytoplasmic IgM$^+$, surface IgM$^+$, diploid (21p$^+$ marker) and FcR$^-$.

Three other independent laboratories have used GM4672 which is a LCL subline of the GM1500 cell developed by Croce *et al.*[27]. Osband *et al.* fused GM4672 with peripheral blood mononuclear cells immunized *in vitro* with Rh(D)$^-$ erythrocytes[37]. Hybrids secreted 0.01–0.1 μg/ml of IgG specific for Rh(D). Shoenfeld *et al.* fused GM4672 with blood mononuclear cells stimulated by pokeweed mitogen (PWM). Cells from patients with the autoimmune disorders systemic lupus erythematosus and cold agglutinin disease yielded hybridization frequencies of 17 × 10^{-7} under optimal conditions[38]. Of 108 hybridomas, 16 produced autoantibodies (15%), all of the IgM class. 7 were specific for ssDNA, 6 for platelets and 7 produced cold agglutinins specific for erythrocytes. All hybridomas except the cold agglutinin secreting lines were stable for 7 months and secreted in the range of 1–15 μg/ml IgM antibody. Shoenfeld *et al.* claim that GM4672 may be EBNA$^-$ (Ref. 38) whereas Osband *et al.*[37] do not characterize the line. Since GM4672 is a subline of GM1500 and we are certain that GM1500 is EBNA$^+$ then it is highly likely that GM4672 is also EBNA$^+$

Search for a non-Ig-secreting fusion partner

The presence of secreted Ig molecules synthesized by the parental plasmacytoma cells dilutes the specific antibody of

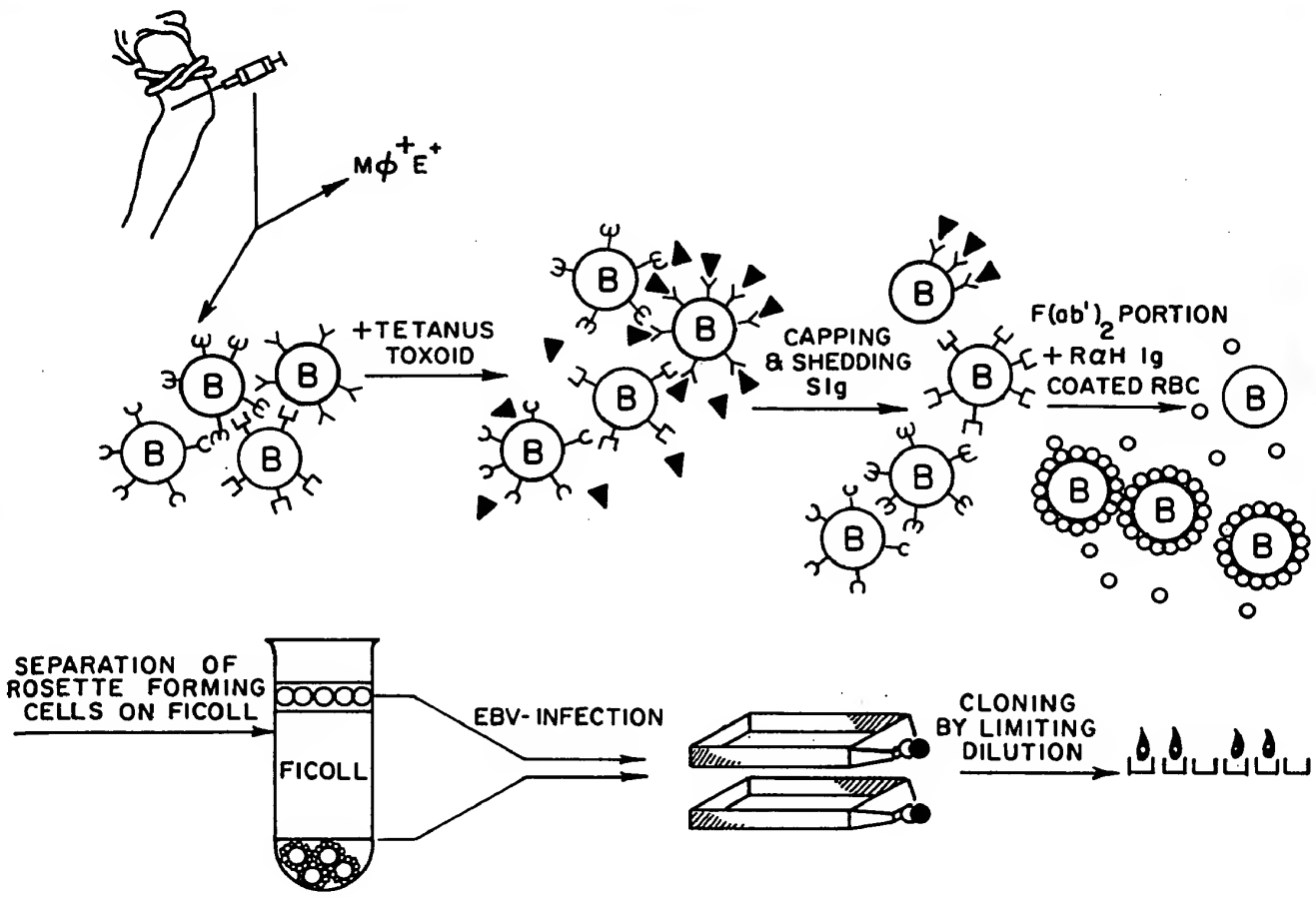

Fig. 1 Enrichment for tetanus-toxoid-binding B cells by negative pre-selection.
This technique was first performed in the mouse by Walker *et al.*[67] and is based on the observation that B cells, upon binding of antigen, usually shed their surface-Ig receptors and become nude (stripped). Those B cells not binding antigen, and therefore maintaining their surface Ig, are removed by rosetting with anti-human Ig-coated SRBC, followed by EBV infection. The established antigen-specific cell line is cloned by limiting dilution on a feeder layer. The B-cell-enriched fraction is obtained by the removal of monocytes (Mϕ+) and cells rosetting with SRBC (E+). (From Ref. 57.)

interest. In the murine system this difficulty was overcome by the development of two kinds of variants; those that synthesize but do not secrete Ig (Refs 39,40) and those that do not synthesize Ig (Ref. 41). Several laboratories are engaged in developing a non-Ig-secreting, HAT-sensitive human myeloma. Unfortunately, the chromosome analysis of the only reported non-Ig-secreting 'human' myeloma fusion partner[24] (RPMI 8226) has revealed it to be a mouse line (Pickering, personal communication).

Sources of donor lymphocytes for f...

To be widel... na technique must wor... lymphocytes and the ... al blood lymphocytes an... eks after a booster inject... circulation capable of ... by producing anti-T1 ... as only 1 × 10⁻⁴ (Ref. ... with a fusion frequency ... approximately 10⁻⁷, makes the chance of obtaining a specific hybrid secreting IgG in the order of 10^{-9}.

In-vitro stimulation by antigen prior to fusion may improve the frequency of positive hybrids: the increase in blasts after immunization was indeed shown to be of predictive value for the successful generation of specific hybrids in the murine system[43]. However, the *in-vitro* stimulation with KLH of peripheral blood lymphocytes from non-boosted donors[44,45] as well as stimulation with KLH and tetanus toxoid of lymphocytes from immunized donors revealed that most of the specific antibody was of the IgM class[46,47]. Nevertheless, *in-vitro* immunized human blood lymphocytes have been fused with KR-4 (Ref. 48) and GM4672 (Ref. 37) to generate human monoclonal antibodies specific for tetanus toxoid and Rh(D), respectively. However, these *in-vitro* immunizations depended on lymphocytes from donors previously immunized with the same antigen. To the best of our knowledge no human hybridoma has yet been constructed with human lymphocytes undergoing a truly primary immune response *in vitro*. This is an important goal and would greatly increase the flexibility of the hybridoma system with respect to the number of antigens which could be used. The current limitation would appear to reside in the poorly developed technology for generating specific human antibody responses *in vitro*. As an alternative, polyclonal activators such as PWM or EBV are known to trigger both IgM and IgG antibody secretion[49], and

therefore can probably be used *in vitro* to expand the numbers of rare antigen-specific B cells of the appropriate isotype prior to fusion. Chiorazzi *et al.* used PWM-stimulated tonsil cells from a donor immunized with tetanus toxoid for fusion with their 0467.3 line[34]. Shoenfeld *et al.* produced data to show that PWM gave a 3-fold increase in hybrids between GM4672 and either blood mononuclear or spleen cells[38].

In a comparative study[48], we have recently found that EBV-transformed human B cells are 36-fold more susceptible to hybridization (fusion frequency approximately 36 $\times$ 10^{-7}), compared with resting lymphocytes (fusion frequency 1.0 $\times$ 1.0^{-7}) and 6-fold to 11-fold compared with PWM-stimulated cells (fusion frequency 6 $\times$ 10^{-7}) or antigen-stimulated cells *in vitro* (fusion frequency 3 $\times$ 10^{-7}). Another advantage of EBV-infected lymphocytes over PWM-stimulated blasts lies in their continuous growth in tissue culture which allows for the flexibility of repeating the fusion experiments at any time.

Many different organs have been used as a source of donor lymphocytes (Table II) including spleen, tonsil, bone marrow, lymph node and peripheral blood but no significant differences in fusion frequencies were noted and in no case was recovery of higher percentages of antigen-specific hybridomas linked to use of a specific organ. However, more comparative studies are required.

The EBV technique

Immortalization

Several years before the application of hybridoma technology to the production of human monoclonal antibodies, human lymphoid lines producing antibody with defined antigenic specificity were established by EBV 'immortalization'[50]. Epstein-Barr virus is a lymphotropic herpesvirus which transforms normal B lymphocytes, making them capable of culture as established lines. Rosen *et al.*[49] demonstrated that direct infection of purified human blood lymphocytes with EBV *in vitro* induced polyclonal secretion of immunoglobulin. Culture supernatants assayed by radioimmunoassay contained a heterogeneous assortment of immunoglobulin isotypes and antibodies specific for various randomly selected antigens. It became obvious then, that if monospecific B cells expressing EBV receptors could be transformed *in vitro* into continuous cell lines by EBV and if these 'immortalized' cells could be triggered to produce antibodies, permanent lines from B lymphocytes might be established which were capable of producing specific antibodies against any appropriate antigen.

Selection of antigen-specific cells

In this technique, as in the hybridoma procedure, it is important to use the blood lymphocytes of individuals who have previously been immunized with the antigens and have increased numbers of specific antibody-producing cells. The procedure involves two steps: (1) the enrichment of cells with receptors for the given antigen; and (2) 'immortalization' of these cells by EBV infection. Preselection of antigen-specific cells may facilitate the establishment of specific cell lines since even after immunization *in vivo*, only a small fraction of the B lymphocytes produce the desired antibody. Several methods of preselection have been tried: (i) antigen-specific lymphocytes were enriched by rosetting with antigen-coupled erythrocytes[50–56]; (ii) fluorescence-labelled antigen was bound to the surface of the antigen-specific cells which were subsequently separated electronically on the fluorescence activated cell sorter (FACS)[57]; and (iii) cells which did not bind antigen were removed[57] (Fig. 1). However, despite the occasional success of these techniques, it is not yet clear which type of B cell can be infected and triggered by EBV to secrete immunoglobulin, and therefore some methods of enrichment for antigen-binding cells may lead to the selection of cells which are at an inappropriate stage of maturation for Ig secretion after EBV transformation under the conditions employed. An alternative strategy involves the transformation of the total B-cell population with subsequent cloning and testing for antibody-producing cultures[58–60].

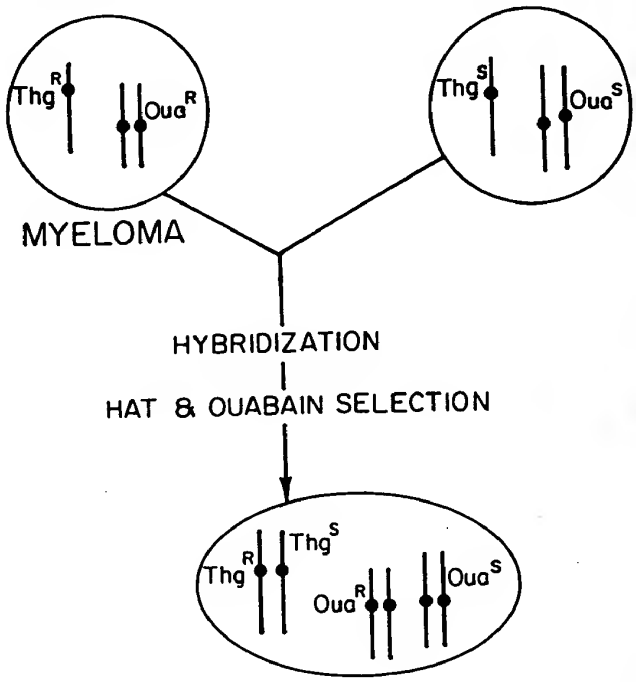

Fig. 2 Basis for hybrid selection in HAT medium containing ouabain.

6-Thioguanine resistance: 6-thioguanine-resistant (Thg^R) cells lack the enzyme HGPRT, which is coded by genes on the X chromosome. There is only one functional X chromosome per cell [♂, XY], [♀, X(X)]. Thg^R cells die in HAT medium and, due to a recessive trait of the Thg^R mutation, only the Thg^S cells and hybrids with the Thg^S phenotype expressed can survive in HAT medium.

Ouabain resistance: ouabain resistance is an autosomal dominant trait, so only cells which carry this mutation, Oua^R, and hybrids will grow in ouabain-containing medium. Oua^S cells die in the presence of ouabain due to the inhibition of plasma membrane Na^+ K^+ -ATPase by this drug.

Characteristics of the system

Using the EBV technique, several cell lines have been established and a variety of antibodies obtained: IgM anti-NNP (4-hydroxy-3,5-dinitrophenacetic acid)[50]; IgM anti-TNP (trinitrophenyl hapten)[51]; IgM anti-streptococcal carbohydrate A (Ref. 52); IgG anti-tetanus toxoid[58,59]; IgG

and IgM anti-Rhesus antigen D[53,54]; IgM anti-phosphorylcholine[60]; IgM anti-IgG complexed with antigens (i.e. rheumatoid factor[55,56]); and IgM anti-tetanus toxoid[57]. The main limitations of the EBV technique are the low quantities of antibody produced (< 1 μg/ml) and the relative instability (< 8 months) of these lines. The reason for the instability of some of these lines is still unclear. In the cell lines which secrete anti-tetanus toxoid (TT), declining anti-TT antibody production was observed in uncloned bulk cultures[57]. The reason for the gradual loss of antibody secretion did not reside solely in differential growth rates of producer and non-producer cells. It is likely that more complex, intracellular changes are involved, such as the selective loss of light-chain expression that we observed in one TT-specific, cloned cell line after 8 months of continuous culture[31]. However, it is possible to rescue high amounts of antibody production in these declining EBV lines by somatic cell hybridization as outlined below.

The EBV-hybridoma technique

Antibody secretion can be 'rescued' in B cells at various stages of maturation. Laskov *et al.*[61] reported the induction of IgM secretion in a murine B lymphoma, which expressed only surface IgM, by fusion with IgG-secreting plasmacytoma cells. Levy and Dilley have demonstrated[62] that human neoplastic B cells which do not normally secrete Ig can be induced to secrete large amounts of Ig when hybridized to mouse plasmacytoma cells.

These results suggested that a general approach to the establishment of human antibody-secreting lines might consist of somatic cell fusion of EBV-transformed cells with a plasmacytoma cell line. First, an EBV-transformed clone (B6) producing anti-tetanus toxoid antibody and obtained from blood lymphocytes of a healthy TT-immunized donor was fused with a non-Ig-producing murine plasmacytoma P3X63Ag.8 v.653 and selected in HAT medium containing 10^{-5} M ouabain (Oua), since the EBV-infected lymphocytes used for fusion were immortalized and could not be counter-selected otherwise (Fig. 2)[5]. Hybrid clones have been stable for 6 months of culture and produced up to 2 μg/ml of specific antibody on days 9 and 10 after seeding, whereas the human EBV parent produced only 0.85 μg/ml. Clones eventually lost antibody production after 6 months of continuous culture, possibly due to the inherent chromosomal instability of interspecies hybrids.

In order to avoid this instability, the EBV-transformed, anti-TT-antibody-producing clone (B6) was fused with a human partner. Because ouabain resistance is a dominant trait and mouse cells have a naturally 10 000-fold greater resistance to it than human cells, ouabain became very useful for selecting mouse × human hybrids. However, this species difference in ouabain sensitivity is of no advantage when both fusion partners are of human origin. Therefore, in order to obtain the human × human hybrids, especially since both parental cells showed indefinite outgrowth *in vitro*, it was necessary to select a ouabain-resistant fusion partner. The 6-thioguanine-resistant GM1500 6TG A-11 human lymphoblastoid cells were mutagenized by low-level gamma irradiation, selected for ouabain resistance and fused with an EBV-transformed cell line producing anti-TT antibody[31]. Owing to the dominance of ouabain resistance and the recessiveness of thioguanine resistance in the parental LCL, now designated KR-4, only hybrid cells derived from the fusion of KR-4 and the EBV clone could survive selection in HAT medium containing ouabain (Fig. 2). The hybridomas produced 4-fold to 8-fold more anti-TT antibody per 10^6 cells than did the EBV parent. Biosynthetic labelling of proteins followed by SDS-PAGE of immunoprecipitates revealed that hybrids synthesized both μ and λ chains from the parental lines as well as κ light chains. The EBV-transformed parental line synthesized very little light-chain in comparison to μ chain, a finding which may explain the declining anti-TT titer observed during prolonged proliferation. It is noteworthy that anti-TT production in hybridomas remained stable throughout 12 months of continuous culture to the present time. Indeed, the karyotype analysis of human × human hybrids indicated that chromosome segregation was very limited, and in several instances a chromosome model number close to the tetraploid number was found.

Therefore, cell fusion of EBV lines and human lymphoblastoid or plasmacytoma cell lines in the future are likely to yield hybrids that retain the advantageous features of each system while overcoming their pitfalls. It is conceivable that the hybrid cells will be successfully passed into nude mice depleted of natural killer cells; thus solving the problem of bulk production.

The advantages of human monoclonal antibodies

Human monoclonal antibodies are desirable and have advantages over the conventional murine fusion products for several reasons: (i) Human monoclonal antibodies are preferable for γ-globulin therapy because of the risk of sensitization with xenoantisera. Almost half of the twenty patients treated to date in various centres with murine monoclonal antibodies have developed an antibody response to the mouse Ig which prevented effective treatment (Refs 63–66; H. Sears, B. Atkinson, D. Herlyn *et al.*, unpublished observations). Human Ig would be far less immunogenic in humans than xenogeneic mouse Ig. In the past γ-globulin prophylaxis for infections (tetanus, rabies) was switched from horse antiserum to human antiserum, which elicits far fewer adverse reactions. Even human Ig, however, may be expected in some cases to stimulate a response to allotypic or idiotypic sites. (ii) Autoantibodies or naturally occurring human antibodies could be used as antigens to select and develop human monoclonal anti-idiotypic antibodies, which would potentially be useful for suppressing the response to auto-antigens or transplant antigens. (iii) The human immune response would generate a wider range of antibodies against HLA and other polymorphic surface determinants than immunization across species barriers. (iv) From the biological standpoint, human monoclonal antibodies would tell us more than murine monoclonal antibodies about the spectrum of the human B-cell specificity repertoire. However, the difficulties encountered in the murine hybridoma field are relevant to the human system as well.

Possible limitations

At present human × human hybrids cannot be grown

as ascites tumors in mice, a manipulation which would increase the yield of antibody by over 1 000-fold. Experiments are underway in several laboratories to solve the problem by using nude mice and immunosuppressive treatments designed especially to inactivate natural killer (NK) cells.

The other potential limitation relates to the presence of either EBV or retroviruses in monoclonal antibody preparations intended for human use. Xenotropic retroviruses are released from many murine hybridomas or fusion partners and are known to be infectious from human cells[68]. This knowledge, however, has not prevented the experimental use of murine monoclonal antibodies in man (Refs 63–66; H. Sears, B. Atkinson, D. Herlyn *et al.*, unpublished observations). Similar C-type and A-type virus particles have not yet been observed in human fusion partners or hybridomas[28], but the recent discovery of a human C-type virus, HTLV, in certain T-cell lymphomas[69,70] warrants a closer look at human fusion partners of the B-cell lineage.

The EBV used for human hybridoma work is derived from the B95-8 marmoset cell line[71]. This virus transforms human B lymphocytes *in vitro* and the EBV nuclear antigen, EBNA, is expressed but the viral cycle is not completed. Consequently infectious virus is not released, although the possibility of contaminating hybridoma supernatants with transforming viral DNA does exist at least in theory. However, virus and viral DNA can easily be inactivated or removed from antibody preparations which can be monitored by sensitive B-cell transformation tests and possibly by injection into marmosets, a species in which EBV is rapidly fatal. By analogy, hyperimmune serum from hepatitis patients is currently used for γ-globulin prophylaxis after removal of contaminating virus. As an additional safeguard, potential recipients of human monoclonal antibodies could be screened for serum antibodies to EBV. Most adults in Western countries are positive, having been exposed to infectious mononucleosis. Only in the very rare X-linked lymphoproliferative syndrome[72] would EBV infection be life-threatening. Some patients have already been exposed to EBV-carrying human hybridomas growing in patients within implanted, cell-impermeable chambers (K. Sikora, personal communication). As in all novel therapies, the potential benefits to the patient will have to be weighed against any potential risks. If certain monoclonal antibodies should prove efficacious in life-threatening human diseases, then a decision not to adopt them for widespread use because of unproven risks becomes ethically indefensible.

Conclusions

10 human fusion partners have been described for the production of human hybridomas. The majority are lymphoblastoid cell lines and only 2 are definite plasmacytomas. A detailed comparison of hybridization frequencies, yield of antigen-specific hybridomas, immunoglobulin secretion levels, cloning efficiencies, division times and stability does not allow one to choose a clearly superior fusion partner and does not allow any generalizations as to whether LCL or plasmacytomas are better fusion partners. However, in several cases efficiency approaches that

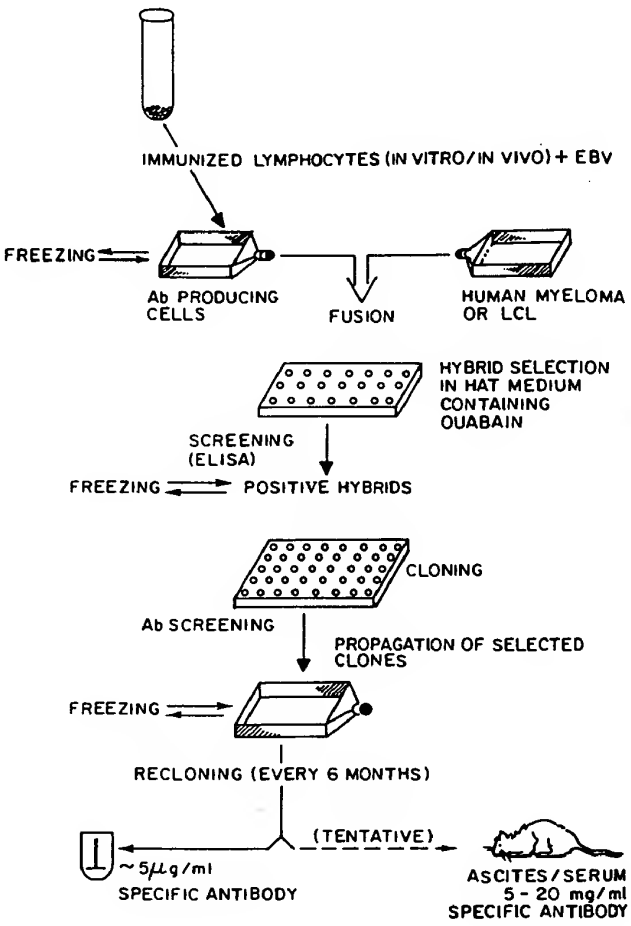

Fig. 3 Construction of human × human hybridomas with EBV-transformed cell lines.

B cells from the peripheral blood of an immunized donor or patient are infected with EBV, screened for specific antibody production and subsequently fused with a human plasmacytoma or LCL. After selection in HAT medium containing ouabain the hybridomas are tested for specific antibody production by ELISA, the positive hybrids are cloned, recloned and then propagated in bulk culture or (tentatively) in the peritoneal cavity of NK-suppressed nude mice. The number of positive hybrids (for example specific for tetanus toxoid) depends on the specificity of the EBV cell line. If the fusion is done 2–3 weeks after EBV transformation without any selection, then approximately 0.5% of hybrids are positive for a given antibody. If the EBV line is already selected by partial cloning, the number of positive hybrids can increase to 5% of hybrids. (From Refs 31, 48.)

in the murine system with fusion frequencies of $> 35 \times 10^{-7}$ and *in vitro* secretion levels of > 5 μg/ml specific antibody. Lymphocytes from the spleen, lymph nodes, tonsils, bone marrow and peripheral blood of hyperimmune patients or *in vitro* immunized cultures have been fused effectively. Thus far it has not been possible to construct a non-Ig-secreting fusion partner in the human.

A viable alternative to the construction of human hybridomas is the use of EBV to immortalize antibody-secreting B cells of chosen specificity. Many[11] human monoclonal antibodies have been obtained in this way. However, yields of Ig (< 0.5 μg/ml) and long-term stability (< 6 months) are often low. One solution has been to fuse EBV-transformed cultures with a HAT-sensitive, ouabain-resistant LCL to efficiently rescue high levels (5

μg/ml) of stable (> 1 year) antibody production. This EBV-hybridoma technique (Fig. 3) offers a high degree of flexibility since the use of EBV (i) immortalizes the donor B cells for future use and repeated fusions; (ii) aids the expansion of rare antigen-specific B cells in the peripheral blood prior to fusion; and (iii) increases hybridization frequencies over 10-fold. One limitation of the system is that only 1 of 21 hybridomas obtained in this way have secreted antigen-specific IgG; most (20/21) produce IgM.

Human monoclonal antibodies produced by the EBV, hybridoma or EBV-hybridoma technique are potential tools for the diagnosis and treatment of human disease. However, before this potential is realized, two major problems must be resolved. One is the bulk production of monoclonal antibodies by growth of hybridomas as ascites tumors in experimental animals. The other is the presence of either EBV or C-type virus in monoclonal antibody preparations intended for human use.

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Continuous cultures of fused cells secreting antibody of predefined specificity

THE manufacture of predefined specific antibodies by means of permanent tissue culture cell lines is of general interest. There are at present a considerable number of permanent cultures of myeloma cells[1,2] and screening procedures have been used to reveal antibody activity in some of them. This, however, is not a satisfactory source of monoclonal antibodies of predefined specificity. We describe here the derivation of a number of tissue culture cell lines which secrete anti-sheep red blood cell (SRBC) antibodies. The cell lines are made by fusion of a mouse myeloma and mouse spleen cells from an immunised donor. To understand the expression and interactions of the Ig chains from the parental lines, fusion experiments between two known mouse myeloma lines were carried out.

Each immunoglobulin chain results from the integrated expression of one of several V and C genes coding respectively for its variable and constant sections. Each cell expresses only one of the two possible alleles (allelic exclusion; reviewed in ref. 3). When two antibody-producing cells are fused, the products of both parental lines are expressed[4,5], and although the light and heavy chains of both parental lines are randomly joined, no evidence of scrambling of V and C sections is observed[4]. These results, obtained in an heterologous system involving cells of rat and mouse origin, have now been confirmed by fusing two myeloma cells of the same mouse strain, and provide the background for the derivation and understanding of antibody-secreting hybrid lines in which one of the parental cells is an antibody-producing spleen cell.

Two myeloma cell lines of BALB/c origin were used. P1Bul is resistant to 5-bromo-2′-deoxyuridine[6], does not grow in selective medium (HAT, ref. 6) and secretes a myeloma protein, Adj PC5, which is an IgG2A (κ), (ref. 1). Synthesis is not balanced and free light chains are also secreted. The second cell line, P3-X63Ag8, prepared from P3 cells[8], is resistant to 20 μg ml^{-1} 8-azaguanine and does not grow in HAT medium. The protein secreted (MOPC 21) is an IgG1 (κ) which has been fully sequenced[7,8]. Equal numbers of cells from each parental line were fused using inactivated Sendai virus[9] and samples contining 2×10^5 cells were grown in selective medium in separate dishes. Four out of ten dishes showed growth in selective medium and these were taken as independent hybrid lines, probably derived from single fusion events. The karyotype of the hybrid cells after 5 months in culture was just under the sum of the two parental lines (Table 1). Figure 1 shows the isoelectric focusing[10] (IEF) pattern of the secreted products of different lines. The hybrid cells (samples *c–h* in Fig. 1) give a much more complex pattern than either parent (*a* and *b*) or a mixture of the parental lines (*m*). The important feature of the new pattern is the presence of extra bands (Fig. 1, arrows). These new bands, however, do not seem to be the result of differences in primary structure; this is indicated by the IEF pattern of the products after reduction to separate the heavy and light chains (Fig. 1*B*). The IEF pattern of chains of the hybrid clones (Fig. 1*B*, *g*) is equivalent to the sum of the IEF pattern (*a* and *b*) of chains of the parental clones with no evidence of extra products. We conclude that, as previously shown with interspecies hybrids[4,5], new Ig molecules are produced as a result of mixed association between heavy and light chains from the two parents. This process is intracellular as a mixed cell population does not give rise to such hybrid molecules (compare *m* and *g*, Fig. 1*A*). The individual cells must therefore be able to express both isotypes. This result shows that in hybrid cells the expression of one isotype and idiotype does not exclude the expression of another: both heavy chain isotypes (γ1 and γ2a) and both V_H and both V_L regions (idiotypes) are expressed. There are no allotypic markers for the C_K region to provide direct proof for the expression of both parental C_K regions. But this is indicated by the phenotypic link between the V and C regions.

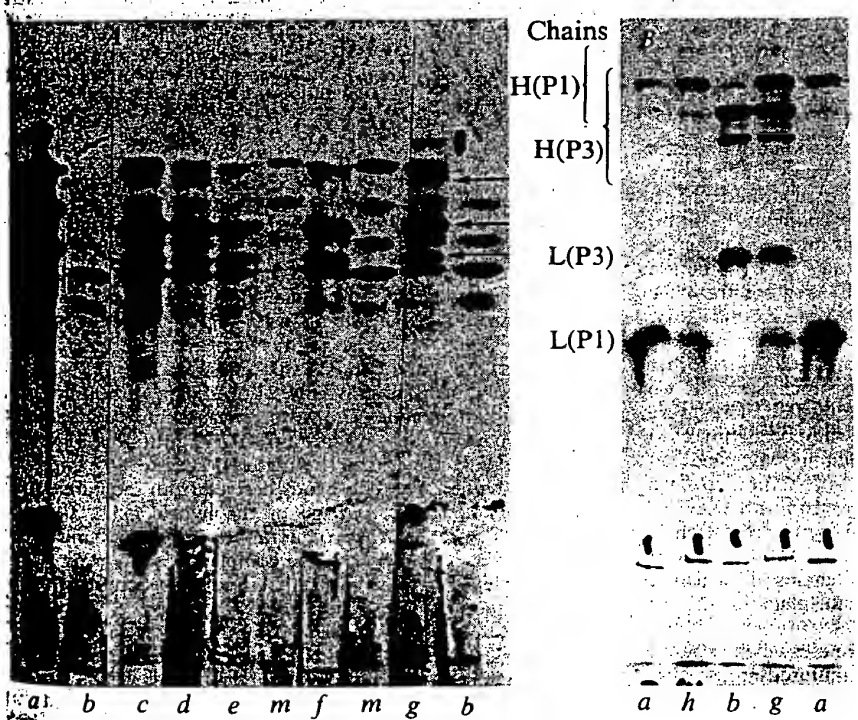

Fig. 1 Autoradiograph of labelled components secreted by the parental and hybrid cell lines analysed by IEF before (*A*) and after reduction (*B*). Cells were incubated in the presence of ^{14}C-lysine[14] and the supernatant applied on polyacrylamide slabs. *A*, *p*H range 6.0 (bottom) to 8.0 (top) in 4 M urea. *B*, *p*H range 5.0 (bottom) to 9.0 (top) in 6 M urea; the supernatant was incubated for 20 min at 37 °C in the presence of 8 M urea, 1.5 M mercaptoethanol and 0.1 M potassium phosphate *p*H 8.0 before being applied to the right slab. Supernatants from parental cell lines in: *a*, P1Bul; *b*, P3-X67Ag8; and *m*, mixture of equal number of P1Bul and P3-X67Ag8 cells. Supernatants from two independently derived hybrid lines are shown: *c–f*, four subclones from Hy-3; *g* and *h*, two subclones from Hy-B. Fusion was carried out[4,9] using 10^6 cells of each parental line and 4,000 haemagglutination units inactivated Sendai virus (Searle). Cells were divided into ten equal samples and grown separately in selective medium (HAT medium, ref. 6). Medium was changed every 3 d. Successful hybrid lines were obtained in four of the cultures, and all gave similar IEF patterns. Hy-B and Hy-3 were further cloned in soft agar[14]. L, Light; H, heavy.

Figure 1*A* shows that clones derived from different hybridisation experiments and from subclones of one line are indistinguishable. This has also been observed in other experiments (data not shown). Variants were, however, found in a survey of 100 subclones. The difference is often associated with changes

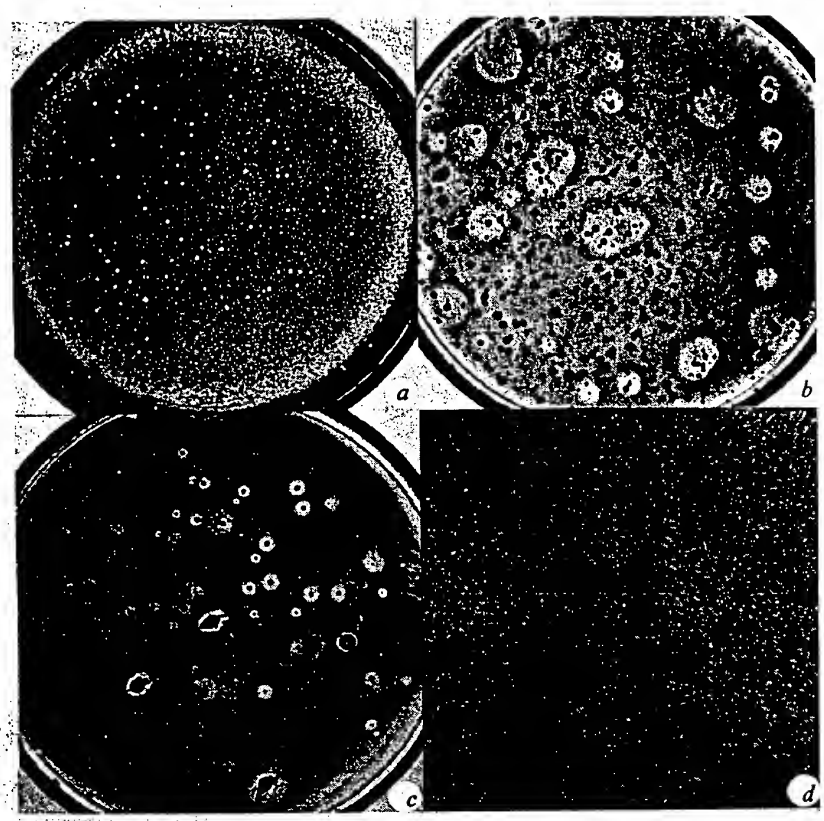

Fig. 2 Isolation of an anti-SRBC antibody-secreting cell clone. Activity was revealed by a halo of haemolysed SRBC. Direct plaques given by: *a*, 6,000 hybrid cells Sp-1; *b*, clones grown in soft agar from an inoculum of 2,000 Sp-1 cells; *c*, recloning of one of the positive clones Sp-1/7; *d*, higher magnification of a positive clone. Myeloma cells (10^7 P3-X67Ag8) were fused to 10^8 spleen cells from an immunised BALB/c mouse. Mice were immunised by intraperitoneal injection of 0.2 ml packed SRBC diluted 1:10, boosted after 1 month and the spleens collected 4 d later. After fusion, cells (Sp-1) were grown for 8 d in HAT medium, changed at 1–3 d intervals. Cells were then grown in Dulbecco modified Eagle's medium, supplemented for 2 weeks with hypoxanthine and thymidine. Forty days after fusion the presence of anti-SRBC activity was revealed as shown in *a*. The ratio of plaque forming cells/total number of hybrid cells was 1/30. This hybrid cell population was cloned in soft agar (50% cloning efficiency). A modified plaque assay was used to reveal positive clones shown in *b*–*d* as follows. When cell clones had reached a suitable size they were overlaid in sterile conditions with 2 ml 0.6% agarose in phosphate-buffered saline containing 25 µl packed SRBC and 0.2 ml fresh guinea pig serum (absorbed with SRBC) as source of complement. *b*, Taken after overnight incubation at 37 °C. The ratio of positive/total number of clones was 1/33. A suitable positive clone was picked out and grown in suspension. This clone was called Sp-1/7, and was recloned as shown in *c*; over 90% of the clones gave positive lysis. A second experiment in which 10^6 P3-X67Ag8 cells were fused with 10^8 spleen cells was the source of a clone giving rise to indirect plaques (clone Sp-2/3-3). Indirect plaques were produced by the addition of 1:20 sheep anti-MOPC 21 antibody to the agarose overlay.

in the ratios of the different chains and occasionally with the total disappearance of one or other of the chains. Such events are best visualised on IEF analysis of the separated chains (for example, Fig. 1*h*, in which the heavy chain of P3 is no longer observed). The important point that no new chains are detected by IEF complements a previous study[4] of a rat–mouse hybrid line in which scrambling of *V* and *C* regions from the light chains of rat and mouse was not observed. In this study, both light chains have identical C_κ regions and therefore scrambled V_L–C_L molecules would be undetected. On the other hand, the heavy chains are of different subclasses and we expect scrambled V_H–C_H to be detectable by IEF. They were not observed in the clones studied and if they occur must do so at a lower frequency. We conclude that in syngeneic cell hybrids (as well as in interspecies cell hybrids) *V*–*C* integration is not the result of cytoplasmic events. Integration as a result of DNA translocation or rearrangement during transcription is also suggested by the presence of integrated mRNA molecules[11] and by the existence of defective heavy chains in which a deletion of *V* and *C* sections seems to take place in already committed cells[12].

The cell line P3-X63Ag8 described above dies when exposed to HAT medium. Spleen cells from an immunised mouse also die in growth medium. When both cells are fused by Sendai virus and the resulting mixture is grown in HAT medium, surviving clones can be observed to grow and become established after a few weeks. We have used SRBC as immunogen, which enabled us, after culturing the fused lines, to determine the presence of specific antibody-producing cells by a plaque assay technique[13] (Fig. 2*a*). The hybrid cells were cloned in soft agar[14] and clones producing antibody were easily detected by an overlay of SRBC and complement (Fig. 2*b*). Individual clones were isolated and shown to retain their phenotype as almost all the clones of the derived purified line are capable of lysing SRBC (Fig. 2*c*). The clones were visible to the naked eye (for example, Fig. 2*d*). Both direct and indirect plaque assays[13] have been used to detect specific clones and representative clones of both types have been characterised and studied.

The derived lines (Sp hybrids) are hybrid cell lines for the following reasons. They grow in selective medium. Their karyotype after 4 months in culture (Table 1) is a little smaller than the sum of the two parental lines but more than twice the chromosome number of normal BALB/c cells, indicating that the lines are not the result of fusion between spleen cells. In addition the lines contain a metacentric chromosome also present in the parental P3-X67Ag8. Finally, the secreted immunoglobulins contain MOPC 21 protein in addition to new unknown components. The latter presumably represent the chains derived from the specific anti-SRBC antibody. Figure 3 shows the IEF pattern of the material secreted by two such Sp hybrid clones. The IEF bands derived from the parental P3 line are visible in the pattern of the hybrid cells, although obscured by the presence of a number of new bands. The pattern is very complex, but the complexity of hybrids of this type is likely to result from the random recombination of chains (see above, Fig. 1). Indeed, IEF patterns of the reduced material secreted by the spleen–P3 hybrid clones gave a simpler pattern of Ig chains. The heavy and light chains of the P3 parental line became prominent, and new bands were apparent.

The hybrid Sp-1 gave direct plaques and this suggested that it produces an IgM antibody. This is confirmed in Fig. 4 which shows the inhibition of SRBC lysis by a specific anti-Ig

Table 1 Number of chromosomes in parental and hybrid cell lines

Cell line	Number of chromosomes per cell	Mean
P3-X67Ag8	66,65,65,65,65	65
P1Bul	Ref. 4	53
Mouse spleen cells	—	40
Hy-B (P1–P3)	112,110,104,104,102	106
Sp-1/7-2	93,90,89,89,87	90
Sp-2/3-3	97,98,96,96,94,88	95

antibody. IEF techniques usually do not reveal 19S IgM molecules. IgM is therefore unlikely to be present in the unreduced sample *a* (Fig. 3*B*) but μ chains should contribute to the pattern obtained after reduction (sample *a*, Fig. 3*A*).

The above results show that cell fusion techniques are a powerful tool to produce specific antibody directed against a predetermined antigen. It further shows that it is possible to isolate hybrid lines producing different antibodies directed against the same antigen and carrying different effector functions (direct and indirect plaque).

The uncloned population of P3–spleen hybrid cells seems quite heterogeneous. Using suitable detection procedures it should be possible to isolate tissue culture cell lines making different classes of antibody. To facilitate our studies we have used a myeloma parental line which itself produced an Ig. Variants in which one of the parental chains is no longer expressed seem fairly common in the case of P1–P3 hybrids (Fig. 1*h*). Therefore selection of lines in which only the specific antibody chains are expressed seems reasonably simple. Alternatively, non-producing variants of myeloma lines could be used for fusion.

We used SRBC as antigen. Three different fusion experiments were successful in producing a large number of antibody-producing cells. Three weeks after the initial fusion, 33/1,086 clones (3%) were positive by the direct plaque assay. The cloning efficiency in the experiment was 50%. In another experiment, however, the proportion of positive clones was considerably lower (about 0.2%). In a third experiment the hybrid population was studied by limiting dilution analysis. From 157 independent hybrids, as many as 15 had anti-SRBC activity. The proportion of positive over negative clones is remarkably high. It is possible that spleen cells which have been triggered during immunisation are particularly successful in giving rise to viable hybrids. It remains to be seen whether similar results can be obtained using other antigenes.

The cells used in this study are all of BALB/c origin and the hybrid clones can be injected into BALB/c mice to produce solid tumours and serum having anti-SRBC activity. It is possible to hybridise antibody-producing cells from different origins[4,5]. Such cells can be grown *in vitro* in massive cultures to provide specific antibody. Such cultures could be valuable for medical and industrial use.

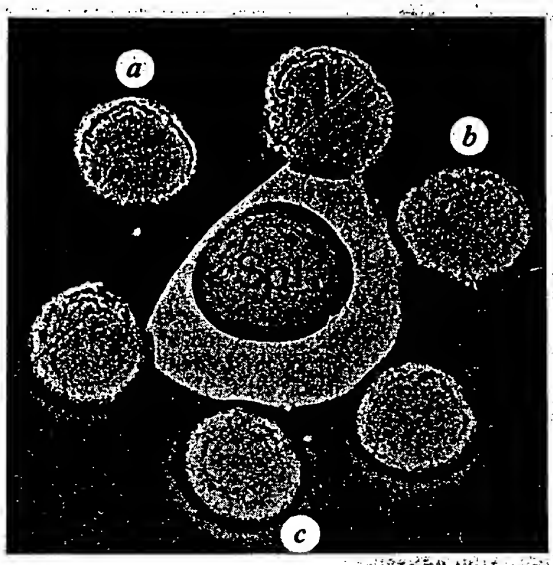

Fig. 4 Inhibition of haemolysis by antibody secreted by hybrid clone Sp-1/7-2. The reaction was in a 9-cm Petri dish with a layer of 5 ml 0.6% agarose in phosphate-buffered saline containing 1/80 (v/v) SRBC. Centre well contains 2.5 μl 20 times concentrated culture medium of clone Sp-1/7-2 and 2.5 μl mouse serum. *a*, Sheep specific anti-mouse macroglobulin (MOPC 104E, Dr Feinstein); *b*, sheep anti-MOPC 21 (P3) IgG1 absorbed with Adj PC-5; *c*, sheep anti-Adj PC-5 (IgG2a) absorbed with MOPC 21. After overnight incubation at room temperature the plate was developed with guinea pig serum diluted 1:10 in Dulbecco's medium without serum.

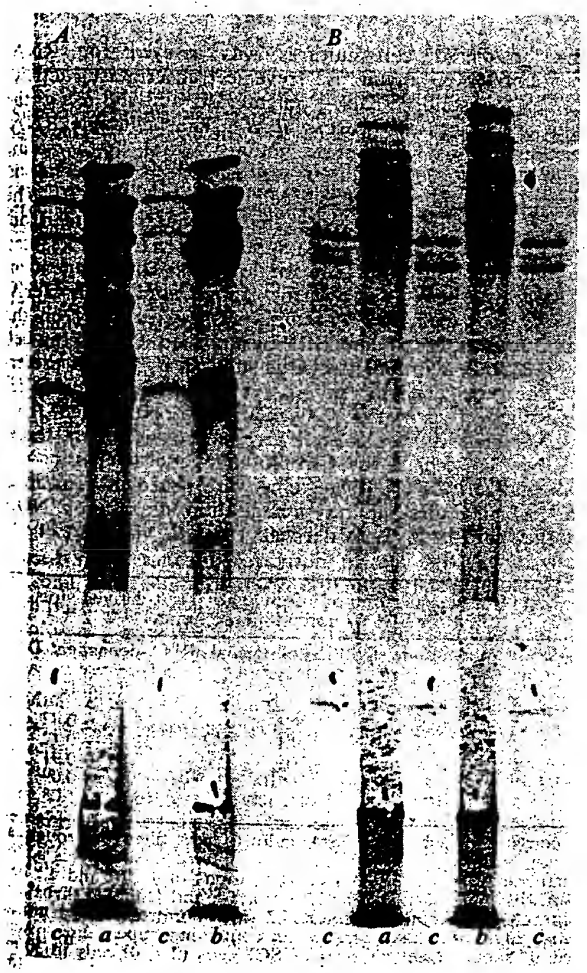

Fig. 3 Autoradiograph of labelled components secreted by anti-SRBC specific hybrid lines. Fractionation before (*B*) and after (*A*) reduction was by IEF. *p*H gradient was 5.0 (bottom) to 9.0 (top) in the presence of 6 M urea. Other conditions as in Fig. 1. Supernatants from: *a*, hybrid clone Sp-1/7-2; *b*, hybrid clone Sp-2/3-3; *c*, myeloma line P3-X67Ag8.

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Research Article

Generation of a Large Combinatorial Library of the Immunoglobulin Repertoire in Phage Lambda

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A novel bacteriophage lambda vector system was used to express in *Escherichia coli* a combinatorial library of Fab fragments of the mouse antibody repertoire. The system allows rapid and easy identification of monoclonal Fab fragments in a form suitable for genetic manipulation. It was possible to generate, in 2 weeks, large numbers of monoclonal Fab fragments against a transition state analog hapten. The methods described may supersede present-day hybridoma technology and facilitate the production of catalytic and other antibodies.

MONOCLONAL ANTIBODIES HAVE BEEN GENERATED THAT catalyze chemical transformations ranging from simple acyl transfer reactions to the energetically demanding hydrolysis of the peptide bond in the presence of metal cofactors (*1, 2–11*). Initially, it was widely held that antibodies would be most useful for catalysis where their predominant role was to overcome entropic barriers that occur along the reaction pathway. The basis of this hypothesis was that the chance occurrence of amino acid side chains capable of acid base catalysis in proximity to the reaction center was unlikely. However, for some reactions, study of the *p*H rate profile has revealed the participation of monobasic residues. Other studies have focused on placing appropriate charges on the antigen to induce specific binding interactions by complementary charged amino acid side chains on the antibody (*9, 12, 13*). Such functionalities might participate as a general acid, base, or nucleophile in the reaction under study.

Apart from the validity of the design of the mechanism based antigen, the probability of finding antibodies where particular amino acid side chains participate in catalysis also depends on the number of different antibodies assayed. Because current methods of generating monoclonal antibodies do not provide for an adequate survey of the available repertoire, we have been devising methods to clone the antibody repertoire in *Escherichia coli* and have described the preparation of a highly diverse immunoglobulin gene library (*14*). Given the difficulty of expressing both heavy and light chains together, we initially considered the construction and expression of libraries restricted to fragments of the variable region of the immunoglobulin (Ig) heavy chain V_H (*14*). In fact, a recent report describes the construction of a plasmid expression library in *E. coli* in which V_H fragments with affinity for keyhole limpet hemocyanin (KLH) and lysozyme have been isolated (*15*). However, the use of isolated V_H fragments as antibody mimics may be limited because (i) the available crystal structures of antibody-antigen complexes show considerable contact between antigen and V_L (light chain

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variable) domain as well as V_H (*16*). More explicitly, in the case of a series of antibodies to dextran, the V_L domain provides contacts critical to antigen binding (*17*). Thus, it is unlikely that the affinity of isolated V_H fragments will generally match that of intact antibodies. (ii) The absence of the V_L domain leaves a large hydrophobic patch on one face of the V_H fragment, which will almost certainly lead to increased nonspecificity relative to whole antibodies (*15*). In contrast, Fab fragments (antigen binding) have been studied for more than 30 years. They behave as whole antibodies in terms of antigen recognition, and their affinity and specificity are well defined. Furthermore, for Fab, the combinatorial properties of heavy and light chains serve as an important source of diversity.

In that individual Fab molecules can be expressed and assembled in *E. coli* (*18*), the route to mimicking the diversity of the antibody system in vitro should lie in solving the problem of expressing the repertoires of heavy and light chains in combination. Accordingly, we used a novel system to enable the construction of bacteriophage lambda (λ) libraries expressing a population of functional antibody fragments (Fab's) with a potential diversity equal to or exceeding that of the parent animal.

Criteria for vector construction. To obtain a vector system for generating the largest number of Fab fragments that could be screened directly, we constructed the expression libraries in bacteriophage λ for the following reasons. First, in vitro packaging of phage DNA is the most efficient method of reintroducing DNA into host cells. Second, it is possible to detect protein expression at the level of single-phage plaques. Finally, in our experience, screens of phage libraries diminish the usual difficulties with nonspecific binding. The alternative, plasmid cloning vectors are only advantageous in the analysis of clones after they have been identified. This advantage is not lost in our system because we use λzap II and are able to excise a plasmid (*19*) containing the heavy chain, light chain or Fab expressing inserts.

The vectors for expression of V_H, V_L, Fv (fragment of the variable region), and Fab sequences are diagrammed in Figs. 1 and 2. They were constructed by a modification of λzap II (*19*) in which we inserted synthetic oligonucleotides into the multiple cloning site. The vectors were designed to be antisymmetric with respect to the Not I and Eco RI restriction sites that flank the cloning and expression sequences. This antisymmetry in the placement of restriction sites in a linear vector such as bacteriophage allows a library expressing light chains to be combined with one expressing heavy chains in order to construct combinatorial Fab expression libraries. The vector λLc1 is designed to serve as a cloning vector for light

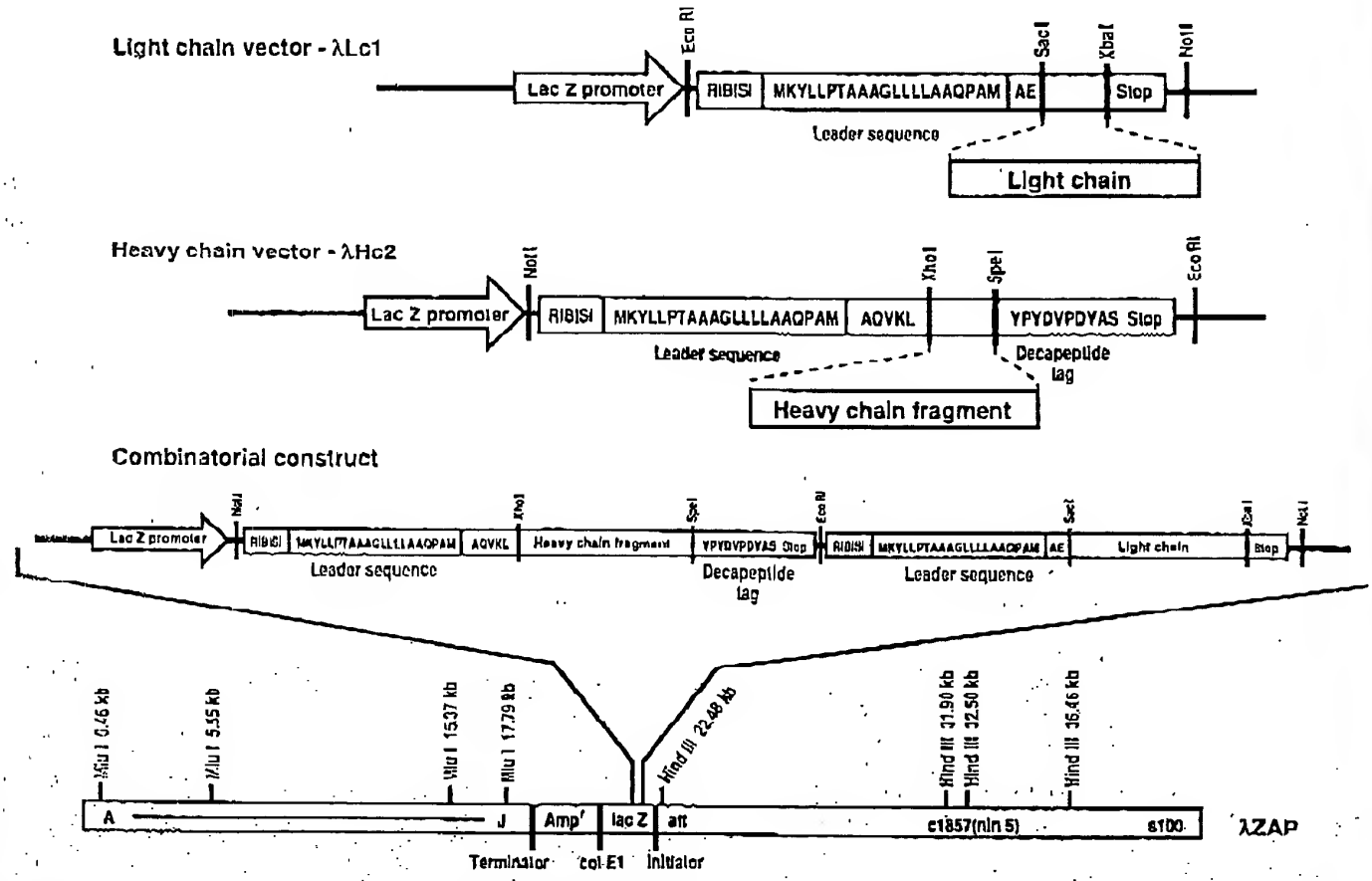

Fig. 1. Combinatorial bacteriophage λ vector system for expression of Fab antibody fragments. The λLc1 vector was constructed for the cloning of PCR amplified products of mRNA that code for light chain protein by inserting the nucleotide sequence shown depicted in Fig. 2A into the Sac I and Xho I sites of λzap II. The sequence was constructed from overlapping synthetic oligonucleotides varying in length from 25 to 50 nucleotides. The λHc2 vector was constructed for cloning PCR amplified products coding for heavy chain Fd sequences by inserting the nucleotide sequence (Fig. 2B) into the Not I and Xho I sites of λzap II. As with the light chain vector, the inserted sequence was constructed from overlapping synthetic oligonucleotides. The combinatorial constructs that can express Fab fragments are generated by cutting DNA isolated from light and heavy chain libraries at the antisymmetric Eco RI site of each vector, followed by re-ligation of the resulting arms. This generates constructs having random combination of light and heavy chains which can be expressed, upon induction with IPTG, from a dicistronic mRNA from the lac Z promoter.

chain fragments, and λHc2 is designed to s as a cloning vector for heavy chain sequences in the initial step of library construction. These vectors are engineered to efficiently clone the products of PCR amplification with specific restriction sites incorporated at each end (*14, 15*). The sequence of the oligonucleotides used to construct these vectors include elements for construction, expression, and secretion of Fab fragments. These oligonucleotides introduce the antisymmetric Not I and Eco RI restriction sites; a leader peptide for the bacterial *pel* B gene, which has previously been successfully used in *E. coli* to secrete Fab fragments (*18*); a ribosome binding site at the optimal distance for expression of the cloned sequence; cloning sites for either the light or heavy chain PCR product; and, in λHc2, a decapeptide tag at the carboxyl terminus of the expressed heavy chain protein fragment. The sequence of the decapeptide tag was useful because of the availability of monoclonal antibodies to this peptide that were used for immunoaffinity purification of fusion proteins (*20*). The restriction endonuclease recognition sites included in the vectors were Sac I and Xba I in λLc1, and Xho I and Spe I in λHc2. The vectors were characterized by restriction digest analysis and DNA sequencing.

Choice of antigen and amplification of antibody fragments. We constructed the initial Fab expression library from mRNA isolated from a mouse that had been immunized with the KLH-coupled *p*-nitrophenyl phosphonamidate antigen 1 (NPN) (Fig. 3). This antigen was shown by Janda and co-workers (*7*) to be an effective one for the generation of catalytic antibodies. Also, the antibodies for the NPN reaction have been identified and therefore facilitate the implementation of assay systems. Finally, successful generation of catalytic antibodies generally requires binding to relatively small organic haptens, and it was necessary to test the suitability of our system for such molecules.

The PCR amplification of messenger RNA (mRNA) isolated from spleen cells or hybridomas with oligonucleotides that incorporate restriction sites into the ends of the amplified product can be used to clone and express heavy chain sequences (*14, 15*). This work is now extended to include the amplification of the Fd (V_H – C_H1) and κ chain sequences (Fig. 4) from mouse spleen cells. The oligonucleotide primers used for these amplifications (Tables 1 and 2) are analogous to those that have been successfully used for amplification of V_H sequences (*14*). The set of 5′ primers for heavy chain amplification was identical to those used to amplify V_H, and those for light chain amplification were chosen similarly (*14, 21*). The 3′ primers of heavy (IgG1) and light (κ) chain sequences included the cysteines involved in disulfide bond formation between heavy and light chains. At this stage no primer was constructed to amplify light (λ) chains since they constitute only a small fraction of murine antibodies (*22*). Restriction endonuclease recognition sequences were incorporated into the primers to allow for the cloning of the amplified fragment into a λ phage vector in a predetermined reading frame for expression.

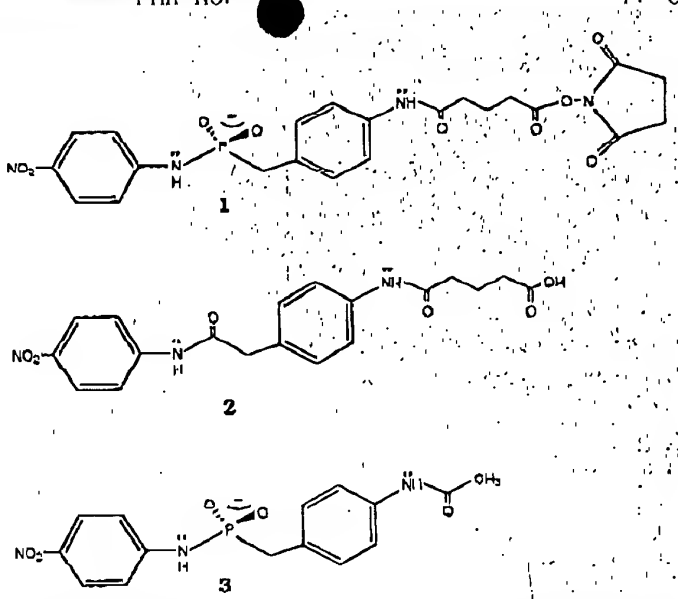

Fig. 3. The transition state analog 1, which induces antibodies for hydrolyzing carboxamide substrate 2. Compound 1 containing a glutaryl spacer and an *N*-hydroxysuccinimide–linker appendage is the form used to couple the hapten 1 to protein carriers KLH and BSA, while 3 is the inhibitor. The phosphonamidate functionality is a mimic of the stereoelectronic features of the transition state for hydrolysis of the amide bond.

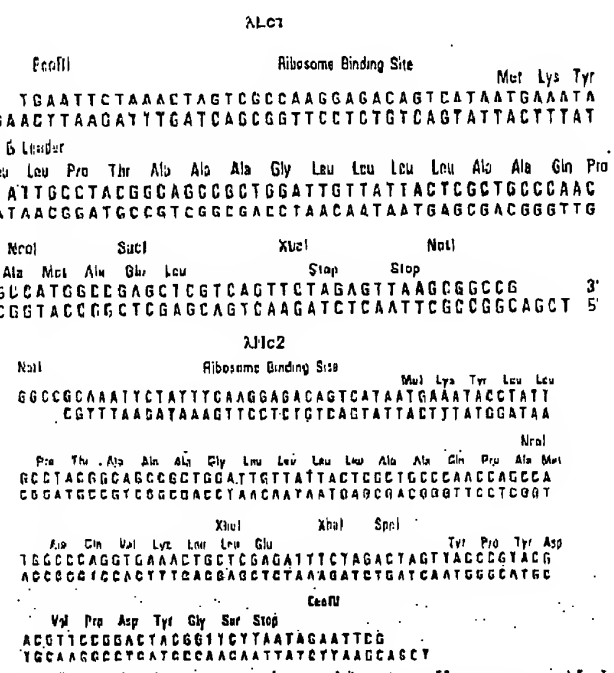

Fig. 2. (A) The nucleotide sequence inserted into λzap II to construct λLc1. (B) The nucleotide sequence inserted into λzap II to construct λHc2.

Library construction. We constructed a combinatorial library in two steps. In the first step, separate heavy and light chain libraries were constructed in λHc2 and λLc1, respectively (Fig. 1). In the second step, these two libraries were combined at the antisymmetric Eco RI sites present in each vector. This resulted in a library of clones each of which potentially coexpresses a heavy and a light chain. The actual combinations are random and do not necessarily reflect the combinations present in the B cell population in the parent animal. The λHc2 expression vector has been used to create a library of heavy chain sequences from DNA obtained by PCR amplification of mRNA isolated from the spleen of a 129 $G1x^+$ mouse previously immunized with NPN-conjugated to KLH. This primary library contains 1.3×10^6 plaque-forming units (pfu) and has been screened for the expression of the decapeptide tag to determine the percentage of clones expressing Fd sequences. The sequence for this peptide is only in frame for expression after the genes for an Fd (or V_H) fragment have been cloned into the vector. At least 80 percent of the clones in the library express Fd fragments when assayed by immunodetection of the decapeptide tag.

The light chain library was constructed in the same way as the heavy chain and shown to contain 2.5×10^6 members. Plaque screening, with an antibody to κ chain, indicated that 60 percent of

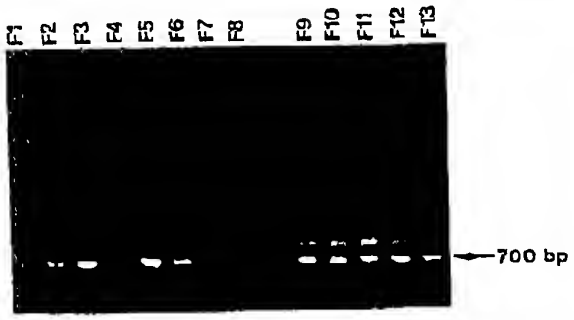

Fig. 4. PCR amplification of Fd and κ regions from the spleen mRNA of a mouse immunized with NPN. Amplification was performed as described (*14*) with RNA-cDNA hybrids obtained by the reverse transcription of the mRNA with primer specific for amplification of heavy chain sequences (12, Table 1) or light chain sequences (9, Table 2). Lanes F1 to F8 represent the product of heavy chain amplification reactions with one of each of the eight 5′ primers (primers 2 to 9, Table 1) and the 3′ primer (primer 12, Table 1). Light chain (κ) amplifications with the 5′ primers (primers 3 to 7, Table 2) and the appropriate 3′ primer (9, Table 2) are shown in lanes F9 through F13). A band of 700 base pairs is seen in all lanes indicating the successful amplification of Fd and κ regions.

HEAVY CHAIN PRIMERS
1) 5′-AGGT(C/G)(C/A)AG(C/A)TG(C/T)TCGAGTC(T/A)GG-3′
2) 5′-AGGTCCAGCTGCTCCACTCTGG-3′
3) 5′-AGGTCCAGCTGCTCGAGTCAGG-3′
4) 5′-AGGTCCAGCTTCTCGAGTCTGG-3′
5) 5′-ACCTCCAGCTTCTCGAGTCAGG-3′
6) 5′-AGGTCCAACTGCTCGAGTCTGG-3′
7) 5′-AGGTCCAACTGCTCGAGTCAGG-3′
8) 5′-AGGTCCAACTTCTCGAGTCTGG-3′
9) 5′-AGGTCCAACTTCTCGAGTCAGG-3′
10) 5′-AGGTIIAICTICTCGAGTC(T/A)GG-3′
11) 5′-CTATTAACTAGTAACGGTAACAGTGGTGCCTTCCCCCA-3′
12) 5′-AGGCTTACTAGTACAATCCCTGGGCACAAT-3′

Table 1. Primers used for amplification of heavy chain Fd fragments for construction of Fab's. Amplification was performed in eight separate reactions, each containing one of the 5′ primers (primers 2 to 9) and one of the 3′ primers (primer 12). The remaining 5′ primers that were used for amplification in a single reaction are either a degenerate primer (primer 1) or a primer that incorporates inosine at four degenerate positions (primer 10). The remaining 3′ primer (primer 11) has been used to construct Fv fragments. The underlined portion of the 5′ primers incorporates an Xho I site and that of the 3′ primer on Spe I restriction site.

Table 2. Primers used for amplification of κ light chain sequences for construction of Fab's. Amplification was performed in five separate reactions, each containing one of the 5′ primers (primers 3 to 7) and one of the 3′ primers (primer 9). The remaining 3′ primer (primer 8) has been used to construct Fv fragments. The underlined portion of the 5′ primers incorporate a Sac I restriction site and that of the 3′ primers an Xba I restriction site.

LIGHT CHAIN PRIMERS
1) 5′-CCAGTTCCGAGCTCGTTGTGACTCAGGAATCT-3′
2) 5′-CCAGTTCCGAGCTCGTGTTGACGCAGCCGCCC-3′
3) 5′-CCAGTTCCGAGCTCGTGCTCACCCAGTCTCCA-3′
4) 5′-CCAGTTCCGAGCTCCAGATGACCCAGTCTCCA-3′
5) 5′-CCAGATGTGAGCTCGTGATGACCCAGACTCCA-3′
6) 5′-CCAGATGTGAGCTCGTCATGACCCAGTCTCCA-3′
7) 5′-CCAGTTCCGAGCTCGTGATGACACAGTCTCCA-3′
8) 5′-GCAGCATTCTAGAGTTTCAGCTCCAGCTTGCC-3′
9) 5′-GCGCCGTCTAGAATTAACACTCATTCCTCTTGAA-3′

the library contained expressed light chain inserts. This relatively small percentage of inserts probably resulted from incomplete dephosphorylation of the vector after cleavage with Sac I and Xba I.

Once obtained, the two libraries were used to construct a combinatorial library by crossing them at the Eco RI site as follows. DNA was first purified from each library. The light chain library was cleaved with Mlu I restriction endonuclease, the resulting 5′ ends were dephosphorylated, and the product was digested with Eco RI. This process cleaved the left arm of the vector into several pieces, but the right arm containing the light chain sequences remained intact. The DNA of heavy chain library was cleaved with Hind III, dephosphorylated, and then cleaved with Eco RI; this process destroyed the right arm, but the left arm containing the heavy chain sequences remained intact. The DNA's so prepared were then mixed and ligated. After ligation, only clones that resulted from combination of a right arm of light chain–containing clones and a left arm of heavy chain–containing clones reconstituted a viable phage. After ligation and packaging, 2.5×10^7 clones were obtained. This is the combinatorial Fab expression library that was screened to identify clones having affinity for NPN. For determining the frequency of the phage clones that coexpress the light and heavy chain fragments, we screened duplicate lifts of the combinatorial library for light and heavy chain expression. In our examination of approximately 500 recombinant phage, approximately 60 percent coexpressed light and heavy chain proteins.

Antigen binding. All three libraries, the light chain, the heavy chain, and Fab were screened to determine whether they contained recombinant phage that expressed antibody fragments binding NPN. In a typical procedure, 30,000 phage were plated and duplicate lifts with nitrocellulose screened for binding to NPN coupled to ^{125}I-labeled bovine serum albumin (BSA) (Fig. 5). Duplicate screens of 90,000 recombinant phage from the light chain library and a similar number from the heavy chain library did not identify any clones that bound the antigen. In contrast, the screen of a similar number of clones from the Fab expression library identified many phage plaques that bound NPN (Fig. 5). This observation indicates that, under conditions where many heavy chains in combination with light chains bind to antigen, heavy or light chains alone do not. Therefore, in the case of NPN, we expect that there are many heavy and light chains that only bind antigen when they are combined with specific light and heavy chains, respectively. This result supports our decision to screen large combinatorial Fab expression libraries. To assess our ability to screen large numbers of clones and obtain a more quantitative estimate of the frequency of antigen binding clones in the combinatorial library, we screened one million phage plaques and identified approximately 100 clones that bound to antigen. For six clones, a region of the plate containing the positive phage plaques and approximately 20 surrounding them was "cored," replated, and screened with duplicate lifts (Fig. 5). As expected, the expression products of approximately 1 in 20 of the phage specifically bind to antigen. Phage which were believed to be negative on the initial screen did not give positives on replating.

To determine the specificity of the antigen-antibody interaction, antigen-binding was subjected to competition with free unlabeled antigen (Fig. 6). These studies showed that individual clones could be distinguished on the basis of antigen affinity. The concentration of free haptens required for complete inhibition of binding varied between 10 to $100 \times 10^{-9} M$, suggesting that the expressed Fab fragments had binding constants in the nanomolar range.

In preparation for characterization of the protein products, a plasmid containing the heavy and light chain genes was excised with helper phage (Fig. 7). Mapping of the excised plasmid demonstrated a restriction pattern consistent with incorporation of heavy and light chain sequences. The protein products of one of the clones was

analyzed by enzyme-linked immunosorbent assay (ELISA) and immunoblotting to establish the composition of the NPN binding protein. A bacterial supernatant after IPTG (isopropyl thiogalactoside) induction was concentrated and subjected to gel filtration. Fractions in the molecular size range 40 to 60 kD were pooled, concentrated, and subjected to a further gel filtration separation. ELISA analysis of the eluted fractions (Fig. 8) indicated that NPN binding was associated with a protein of a molecular size of about 50 kD, which contained both heavy and light chains. An immunoblot of a concentrated bacterial supernatant preparation under nonreducing conditions was developed with antibody to decapeptide. This revealed a 50-kD protein band. We have found that the antigen-binding protein can be purified to homogeneity from bacterial supernate in two steps involving affinity chromatography on protein G followed by gel filtration. SDS-PAGE analysis of the protein revealed a single band at ~50 kD under nonreducing conditions and a doublet at ~25 kD under reducing conditions. Taken together, these results are consistent with NPN-binding being a function of Fab fragments in which heavy and light chains are covalently linked by a disulfide bond.

Properties of the in vivo repertoire compared to the phage combinatorial library. Previously we constructed a highly diverse V_H library in *E. coli*. We have now combined heavy and light chain libraries to clone and express assembled and functional Fab fragments of immunoglobulin. A moderately restricted library was prepared because only a limited number of primers was used for polymerase chain reaction (PCR) amplification of Fd sequences. The library is expected to contain only clones expressing κ-γ1 sequences. However, this is not an inherent limitation of the method since the addition of more primers can amplify any antibody class or subclass. Despite this restriction we were able to isolate a large number of clones producing antigen binding proteins.

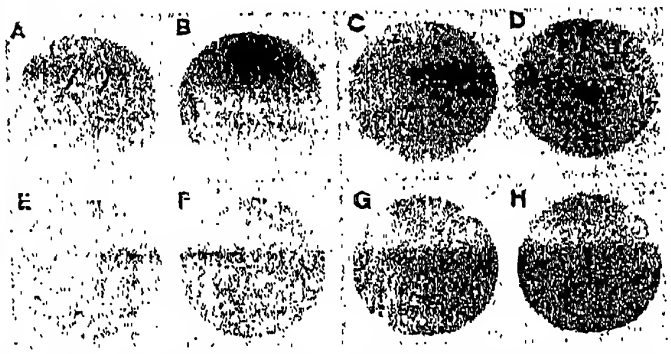

Fig. 5. Screening phage libraries for antigen-binding. Duplicate plaque lifts of Fab (filters A and B), heavy chain (filters E and F), and light chain (filters G and H) expression libraries were screened against ^{125}I-labeled BSA conjugated with NPN at a density of approximately 30,000 plaques per plate. Filters C and D illustrate the duplicate secondary screening of a cored positive from a primary filter A (arrows) as discussed in the text. Standard plaque lift methods were used in screening. Cells (XL1 blue) infected with phage were incubated on 150-mm plates for 4 hours at 37°C; protein expression was induced by overlay with nitrocellulose filters soaked in 1 m*M* IPTG, and the plates were incubated at 25°C for 8 hours. Duplicate filters were obtained during a second incubation under the same conditions. Filters were then blocked in a solution of 1 percent BSA in phosphate-buffered saline (PBS) for 1 hour before incubation (with rocking) at 25°C for 1 hour with a solution of ^{125}I-labeled BSA (at 0.1 μ*M*) conjugated to NPN (2×10^6 cpm/ml; approximately 15 NPN per BSA molecule), in 1 percent BSA in PBS. Background was reduced by preliminary centrifugation of stock ^{125}I-labeled BSA solution at 100,000*g* for 15 minutes and preliminary incubation of solutions with plaque lifts from plates containing bacteria infected with a phage having no insert. After labeling, filters were washed repeatedly with PBS containing 0.05 percent Tween 20 before the overnight development of autoradiographs.

A central issue is how our phage library compares with the in vivo antibody repertoire in terms of size, characteristics of diversity, and ease of access.

The size of the mammalian antibody repertoire is difficult to judge, but a figure of the order of 10^6 to 10^8 different antigen specificities is often quoted. With some of the reservations discussed below, a phage library of this size or larger can readily be constructed by a modification of the method described. Once an initial combinatorial library has been constructed, heavy and light chains can be shuffled to obtain libraries of exceptionally large numbers.

In principle, the diversity characteristics of the naive (unimmunized) in vivo repertoire and corresponding phage library are expected to be similar in that both involve a random combination of heavy and light chains. However, different factors act to restrict the diversity expressed by an in vivo repertoire and phage library. For example, a physiological modification such as tolerance will restrict the expression of certain antigenic specificities from the in vivo repertoire, but these specificities may still appear in the phage library. However, bias in the cloning process may introduce restrictions into the diversity of the phage library. For example, the representation of mRNA for sequences expressed by stimulated B cells can be expected to predominate over those of unstimulated cells because of higher levels of expression. In addition, the resting repertoire might overrepresent spontaneously activated B cells whose immunoglobulins have been suggested to be less specific. In any event, methods exist to selectively exclude such populations of cells. Also, the fortuitous presence of restriction sites in the variable gene similar to those used for cloning and combination will cause them to be eliminated. We can circumvent some of these difficulties by making minor changes, such as introducing amber mutations in the vector system. Different source tissues (for example, peripheral blood, bone marrow, or regional lymph nodes) and different PCR primers (for example, those to amplify different antibody classes), may result in libraries with different diversity characteristics.

Another difference between in vivo repertoire and phage library is

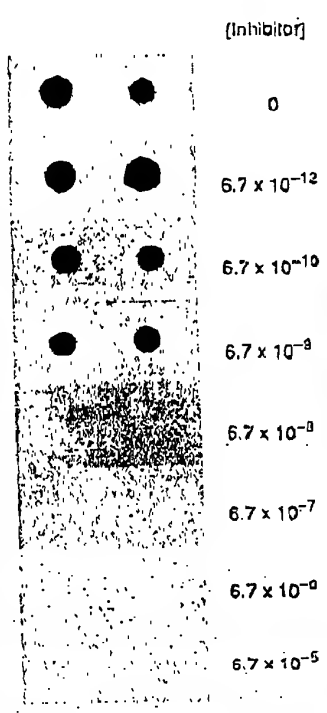

Fig. 6. Specificity of antigen binding shown by competitive inhibition. Filter lifts from positive plaques were exposed to ^{125}I-labeled BSA-NPN in the presence of increasing concentrations of the inhibitor NPN. A number of phages correlated with NPN-binding as in Fig. 5 were spotted in duplicate (about 100 particles per spot) directly onto a bacterial lawn. The plate was then overlaid with an IPTG-soaked filter and incubated for 19 hours at 25°C. The filters were then blocked in 1 percent BSA in PBS before incubation in ^{125}I-BSA–NPN as done previously with the inclusion of varying amounts of NPN in the labeling solution. Other conditions and procedures were as in Fig. 5. The results for a phage of moderate affinity are shown in duplicate in the figure. Similar results were obtained for four other phages with some differences in the effective inhibitor concentration ranges.

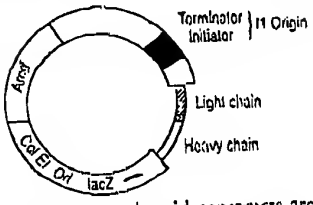

Fig. 7. A plasmid can be excised from λLc1, λHc2, and their combination because they are a modification of λzap II. M13mp8 was used as helper phage and the excised plasmid was infected into a F⁺ derivative of MC1061. The excised plasmid contains the same constructs for antibody fragment expression as do the parent vectors (Fig. 1). These plasmid constructs are more conveniently analyzed for restriction pattern and protein expression of the λ phage clones identified and isolated on the basis of antigen binding. The plasmid also contains an f1 origin of replication which facilitates the preparation of single-stranded DNA for sequence analysis and in vitro mutagenesis.

that antibodies isolated from the repertoire may have benefited from affinity maturation as a result of somatic mutations after combination of heavy and light chains whereas the phage library randomly combines the matured heavy and light chains. Given a large enough phage library derived from a particular in vivo repertoire, the original matured heavy and light chains will be recombined. However, since one of the potential benefits of this technology is to obviate the need for immunization by the generation of a single highly diverse "generic" phage library, it would be useful to have methods to optimize sequences to compensate for the absence of somatic mutation and clonal selection. Three procedures are made readily available through the vector system presented. First, saturation mutagenesis may be performed on the complementarity-determining regions (CDR's) (*23*) and the resulting Fab's can be assayed for increased function. Second, a heavy or a light chain of a clone that binds antigen can be recombined with the entire light or heavy chain libraries, respectively, in a procedure identical to that used to construct the combinatorial library. Third, iterative cycles of the two above procedures can be performed to further optimize the affinity or catalytic properties of the immunoglobulin. The last two procedures are not permitted in B cell clonal selection, which suggests that the methods described here may actually increase our ability to identify optimal sequences.

Access is the third area where it is of interest to compare the in vivo antibody repertoire and phage library. In practical terms the phage library is much easier to access. The screening methods used have allowed one to survey the gene products of at least 50,000 clones per plate so that 10^6 to 10^7 antibodies can be readily examined in a day but the most powerful screening methods depend on selection. In the catalytic antibody system, this may be accomplished by incorporating into the antigen leaving groups necessary for replication of auxotrophic bacterial strains or toxic substituents susceptible to catalytic inactivation. Further advantages are related to the fact that the in vivo antibody repertoire can only be accessed via immunization, which is a selection on the basis of binding affinity. The phage library is not similarly restricted. For example, the only general method to identify antibodies with catalytic properties has been by preselection on the basis of affinity of the antibody to a transition state analog. Such restrictions do not apply to the in vitro library where catalysis can, in principle, be assayed directly. The ability to assay directly large numbers of antibodies for function may allow selection for catalysts in reactions where a mechanism is not well defined or synthesis of the transition state analog is difficult. Assaying for catalysis directly eliminates the bias of the screening procedure for reaction mechanisms limited to a particular synthetic analog; therefore, simultaneous exploration of multiple reaction pathways for a given chemical transformation are possible.

We have described procedures for the generation of Fab fragments that are clearly different in a number of important respects from antibodies. There is undoubtedly a loss of affinity in having monovalent Fab antigen binders, but it is possible to compensate for this by selection of suitably tight binders. For a number of applications such as diagnostics and biosensors, monovalent Fab fragments may be preferable. For applications requiring Fc effector functions, the technology already exists for extending the heavy chain gene and expressing the glycosylated whole antibody in mammalian cells.

Our data show that it is now possible to construct and screen at least three orders of magnitude more clones with monospecificity than previously possible. The data also invite speculation concerning the production of antibodies without the use of live animals.

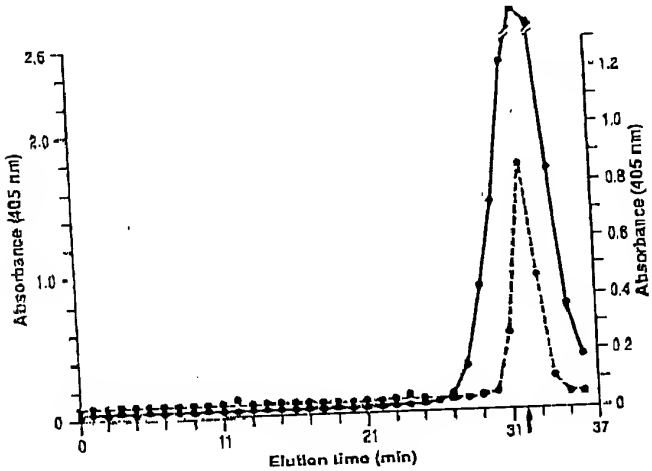

Fig. 8. Characterization of an antigen binding protein. The concentrated partially purified bacterial supernatant of an NPN binding clone was separated by gel filtration and samples from each fraction were applied to microtiter plates coated with BSA-NPN. Addition of either antibody to decapeptide (---) or antibody to κ chain (—, left-hand scale) conjugated with alkaline phosphatase was followed by color development. The arrow indicates the position of elution of a known Fab fragment. The results show that antigen binding is a property of a 50-kD protein containing both heavy and light chains. To permit protein characterization, a single plaque of a NPN-positive clone (Fig. 5) was picked, and the plasmid containing the heavy and light chain inserts (Fig. 7) was excised (*19*). Cultures (500 ml) in L broth were inoculated with 3 ml of a saturated culture of the clone and incubated for 4 hours at 37°C. Protein synthesis was induced by the addition of IPTG to a final concentration of 1 m*M*, and the cultures were incubated for 10 hours at 25°C. The supernatant from 200 ml of cells was concentrated to 2 ml and applied to a TSK-G4000 column. Samples (50 μl) from the eluted fractions were assayed by ELISA. Microtiter plates were coated with BSA-NPN at 1 μg/ml, 50-μl samples were mixed with 50 μl of PBS-Tween 20 (0.05 percent) BSA (0.1 percent) added, and the plates were incubated for 2 hours at 25°C. The plated material was then washed with PBS-Tween 20-BSA and 50 μl of appropriate concentrations of a rabbit antibody to decapeptide (*20*) or a goat antibody to mouse κ light chain (Southern Biotech) conjugated with alkaline phosphatase were added and incubated for 2 hours at 25°C. The plates were again washed, 50 μl of *p*-nitrophenyl phosphate (1 mg/ml in 0.1*M* tris, pH 9.5, containing 50 m*M* $MgCl_2$) was added, and the plates were incubated for 15 to 30 minutes and the absorbance was read at 405 nm.

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22. The Fv (variable region) fragments may be constructed with a 3′ primer that is complementary to the mRNA in the J (joining) region (amino acid 128) and a set of 5′ primers that are complementary to the first strand cDNA in the conserved amino-terminal region of the processed protein.
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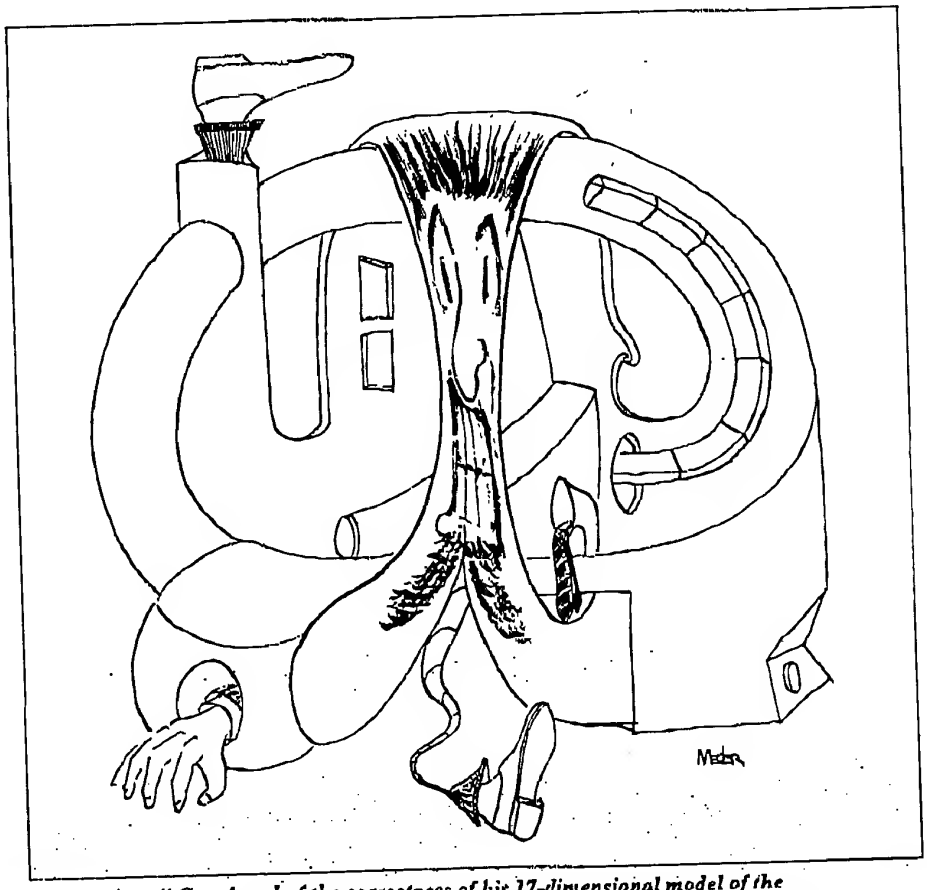

"Convinced of the correctness of his 17-dimensional model of the universe, theorist Martin Nowsky was not above testing it directly."